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THE EFFECTS OF ALTERATIONS IN INTERNAL CA^{2+} LEVELS ON CAT SMALL INTESTINAL SLOW WAVES

BY

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B.S., University of Illinois, 1974
M.S., University of Illinois, 1977

THESIS

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ALLEN WAYNE MANGEL

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FORMAT APPROVAL

To the Graduate College:

The format of the thesis submitted by Allen Wayne Mangel for the degree of Ph.D. is acceptable to the department of Physiology.

April 10, 1980
Date

(Signed)
Departmental Representative

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I. LITERATURE REVIEW

(i) Smooth Muscle

Smooth muscles have been subdivided into two categories: unitary and multiunit smooth muscle. Most visceral smooth muscles are of the unitary type. Unitary muscles are characterized by spontaneous activity, which continues after ganglion degeneration or treatment with neural blockers. The spontaneous activity is, however, modulated by neuronal and hormonal input. Tonic contractions can be induced in some of these muscles by stretch; in others phasic contractile responses occur which are partly myogenic and partly the result of neural stimulation. Conduction of electrical activity is from fiber to fiber, with muscle bundles behaving as electrical syncytia.

In contrast to unitary muscles, multiunit smooth muscles are not spontaneously active but are activated by neural input. Multiunit muscles have poor electrical coupling and are not usually activated by stretch. Rhythmic activity is present only in association with perodic neuronal discharge. The smooth muscles of the eye are the most commonly considered multiunit smooth muscles. Some visceral muscles show both unitary and multiunit properties, that is, are intermediate in type. Invertebrate smooth muscles are mostly under neural control.

Ionic mechanisms responsible for generation of electrical activity in excitable tissues are of two kinds: (1) membrane conductance changes and (2) electrogenic pump activity. To account for spontaneous potential changes in smooth muscle, conductance changes to Na^+, Ca^{2+}, K^+ and Cl^- as well as oscillatory Na^+ and Ca^{2+} pump activity have been postulated at various points in the literature. The study of the ionic basis of rhythmic electrical activity in smooth muscle is limited by the small size of smooth muscle cells (maximum diameter 3-5 μm) and is complicated by the fact that

the same tissue type in different species has (1) different types of electrical activity and (2) different ionic mechanisms responsible for the activity.

Electrical Activity in Smooth Muscle

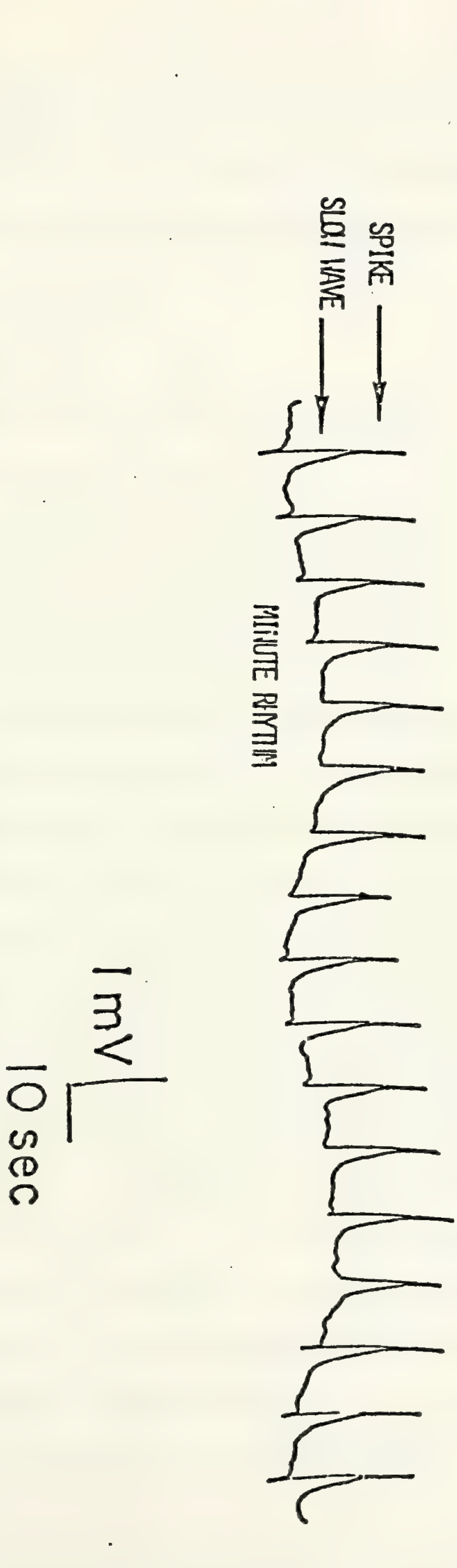
In 1904 Magnus observed that longitudinal fibers of cat small intestinal muscle, separated from the circular fibers, beat rhythmically while isolated circular fibers were quiescent (182,183). According to his observations, the longitudinal muscle carried all the innervation, so he concluded that the source of rhythmicity was neurogenic. However, in 1905 he successfully obtained preparations of denervated intestine which contracted rhythmically (184). In 1914 Gunn and Underhill (123) repeated Magnus' observations. They concluded there could be no doubt about the myogenic basis for the rhythmic contractions.

Alvarez was the first to observe electrical activity in the small intestine (7,8). He showed the occurrence of rhythmic currents in rabbit intestinal segments that were mechanically quiescent. Since that time several types of spontaneous electrical activity have been recorded from the small intestine of various animals: minute rhythms, slow waves, prepotentials, spikes, and action potentials. In addition, activity induced by acetylcholine (ACh) and EGTA has also been recorded.

A. Minute Rhythms

Minute rhythms are rhythmic waves of depolarization with duration approximately one minute (Figure 1). They are 3-20 mV in amplitude (measured intracellularly) and in cat small intestine or dog stomach originate in the longitudinal muscle layer (118,120,189). Minute rhythms are not abolished by removal of Na_o^+ or Ca_o^{2+}, or by application of ouabain, but are eliminated

Figure 1. Extracellular recording (tube electrode) of spikes, slow waves and minute rhythms from intact cat small intestine. The gut is in its normal configuration, i.e., longitudinal muscle facing outward.


SPIKE
SLOW WAVE
MINUTE RHYTHM
1 mV
10 sec

by metabolic inhibitors. In both guinea pig and cat intestine the mechanism responsible for generation of minute rhythms has not been elucidated, but in both cases metabolism appears to play a role in minute rhythm generation (118,189).

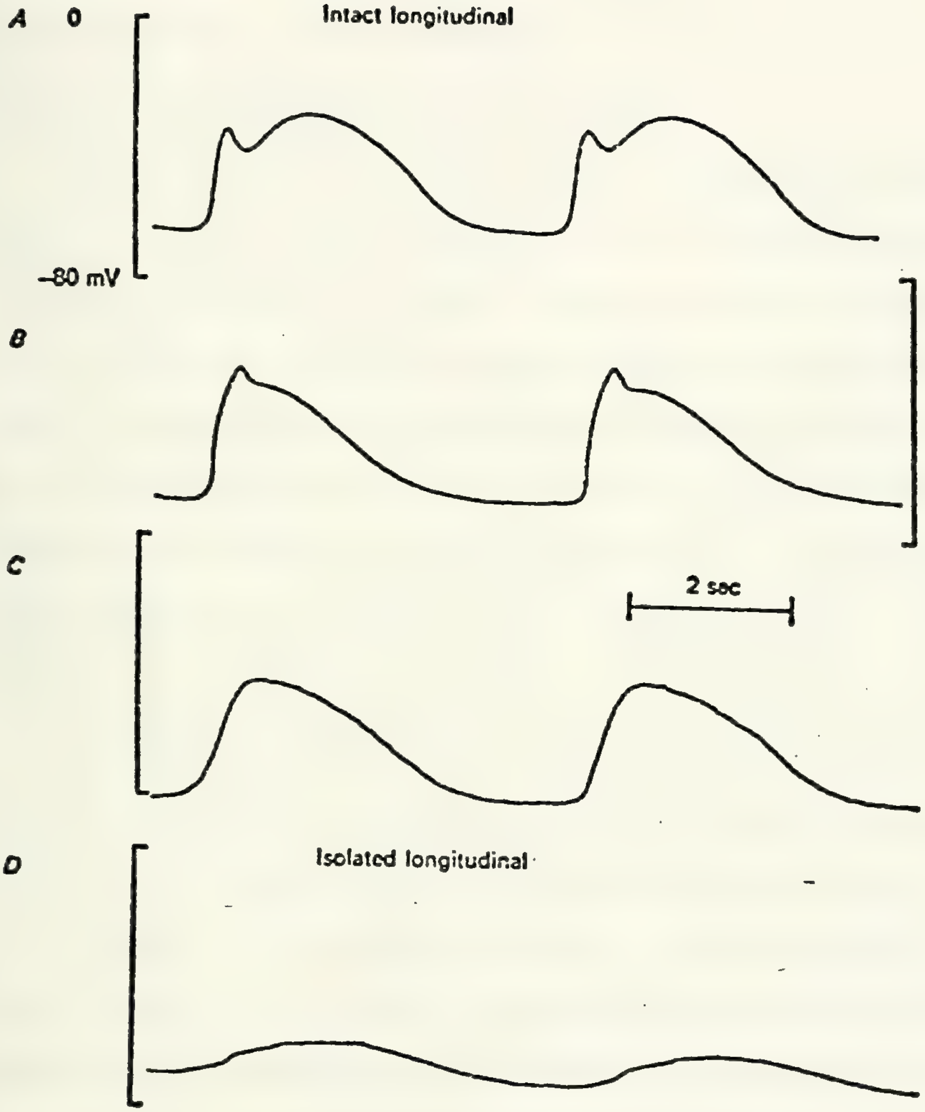
B. Slow Waves

Spontaneous slow potential oscillations have been referred to in the literature as slow waves, basic electrical rhythms, control potentials, electrical control activity, action potentials, first potentials, pacesetter potentials and others. I will use the term slow wave.

Small intestinal slow waves, when measured in intact muscle (i.e., containing both longitudinal and circular muscle layers), occur with a frequency of 7-20 cycles/min and average 27 mV in amplitude (7,29,30,80,89,178) (see Figure 2). Since Magnus (182,183,184) the evidence has supported the longitudinal muscle layer as the site of slow wave generation. Recordings from strips of isolated cat longitudinal muscle in the double sucrose gap, or with micro- and pressure electrodes demonstrate slow wave activity (80,81, 151,178). Recordings from isolated circular muscle do not show slow waves. Not every longitudinal muscle cell generates slow waves but pacemaker regions exist (80,164). The pacemaker regions are approximately 2 mm in diameter and are separated from one another by 3-5 mm, along the longitudinal axis. Taylor *et al*., in studies with rabbit small intestine, suggested that slow waves originate in "hot spots" either between the two muscle layers or in the most peripheral circular muscle, i.e., that closest to the longitudinal muscle layer (283). This has not been supported in studies with cat small intestine.

Recordings from isolated longitudinal muscle of cat show slow waves of identical frequency as intact muscle but reduced amplitude (∿12 mV) and rate

Figure 2. Intracellular recordings of slow waves in cat longitudinal muscle layer. Different shapes are shown in recordings (A-C) from intact segments and (D) from isolated longitudinal muscle. From Connor *et al*., ref. 80.


A
0
Intact longitudinal
-80 mV
B
C
2 sec
D
Isolated longitudinal

of rise (Figure 2) (80). The reduction in slow wave amplitude has been attributed to the removal of the circular muscle layer which amplifies the longitudinal muscle current (77,80). The ionic basis of the amplification is a calcium dependent conductance change.

Between muscle fibers of both the longitudinal and circular muscle layers, of cat small intestine, nexal junctions have been demonstrated (282). No muscle fibers span the region between the muscle layers, but fibrocytes and interstial cells have been observed under electron microscopic observation. The longitudinal and circular muscle layers may be electrically coupled by the fibrocytes and interstial cells (80,282). Slow waves function in coordinating motility in the small intestine by synchronizing spiking in the longitudinal and circular muscle layers (18). This coordination may occur by spread of current through the intermuscle layer connections.

One hypothesis for slow wave propagation suggests that slow waves propagate in the longitudinal muscle layer and are electrotonically conducted in the circular muscle (31,32). Previous evidence (162) and recent studies (77,79) support an alternative hypothesis, that longitudinal muscle pacemakers supply current to the circular muscle where there are regenerative mechanisms of current amplification, which facilitate slow wave propagation.

Cat small intestinal slow waves have been shown to be sensitive to membrane potential changes. Application of steady hyperpolarizing current has been shown to decrease slow wave frequency and increase slow wave amplitude while depolarizing currents have the opposite effect (81). In some quiescent preparations, slow waves can be induced by membrane hyperpolarization while in other cases step or ramp depolarizations from hyperpolarized levels produce slow waves (81). Slow wave frequency can be entrained to

current pulse stimuli whose frequency is faster than the spontaneous rate in isolated longitudinal and intact muscle of cat and rabbit intestine (79, 277). Slow waves can not be entrained to slower than spontaneous frequency even by large current pulses (79).

The small intestine receives both extrinsic and intrinsic innervation. The extrinsic innervation is composed of three parts: excitatory cholinergic, inhibitory adrenergic sympathetic, and nonadrenergic inhibitory innervation (16,47,230). Several studies indicate that there is no effect on slow wave activity from the impulses carried by the parasympathetic nerves to the small intestine (121,204,293), although application of acetylcholine has been shown to alter slow wave frequency (30,184). Stimulation of the sympathetic nerves decreases slow wave frequency in cat small intestine (230).

The intrinsic innervation of the small intestine consists of the myenteric (Auerbach) and submucosal (Meisners) plexuses (16,47,230). The submucosal plexus lies between the muscularis mucosa and the circular muscle layer. Its dendrites sense chemical or mechanical changes in the intestinal lumen. Axons from this plexus synapse on the myenteric plexus. The myenteric plexus lies between the longitudinal and circular muscle and functions as the central coordinator of G.I. nervous activity.

It is generally accepted that slow waves in the small intestine are myogenic rather than neurogenic. Application of cholinergic and adrenergic blocking agents (16,88,91,137), tetrodotoxin (16,230,233), or ganglionic degeneration does not eliminate slow waves (88,230). Neural input may, however, modulate slow wave activity.

Several lines of evidence suggest that slow waves possess a metabolic dependency. Slow waves are reduced in amplitude and may be eliminated by

metabolic inhibitors (152,232). Slow wave frequency exhibits a Q_{10} of 3.25 between 17-27°C and 2.1 between 27-37°C (222). There is a slow wave frequency gradient (oral fast: aboral, slow) corresponding to a gradient of oxygen consumption down the intestine (9). The slow wave frequency gradient has been attributed to this metabolic gradient (9,96). Measurements of NADH fluorescence have been correlated with simultaneously recorded slow waves (78). Averaging of 30-70 slow waves produces a pattern with fluorescence oscillations in phase with electrical oscillations. Changes in fluorescence levels were well correlated with changes in slow wave amplitude.

Rabbit jejenal slow waves are reduced in frequency by detergents (281). Application of 6 µg/ml triton X-100 or 50 µg/ml DOC reversibly decreases slow wave frequency to between 30 to 40% of control levels. High levels of detergent (100 µg/ml triton X-100) irreversibly eliminates slow wave activity. Reduction in external calcium (Ca_o^{2+}) produces a decrease in cat small intestinal slow wave frequency (80). The frequency decline is characterized by an elongation of the diastolic (hyperpolarized) phase of the slow wave (Figure 3).

Slow wave activity is also altered by application of hormones (218). Application of pentagastrin (5×10^{-7} g/ml) and tetragastrin (2.5×10^{-6} g/ml) decrease slow wave frequency. High levels of pancreozymin (5×10^{-6} g/ml) eliminates slow waves while secretin has no effect on frequency regardless of concentration.

The various ionic mechanisms proposed to account for slow wave general are considered later.

C. Prepotentials

Spontaneous spikes (section D) recorded from guinea pig taenia coli,

Figure 3. Intracellular recording of slow waves in isolated cat longitudinal muscle. Region A represents the systolic or depolarized phase of the slow wave; region B is the diastolic or hyperpolarized phase of the slow wave.

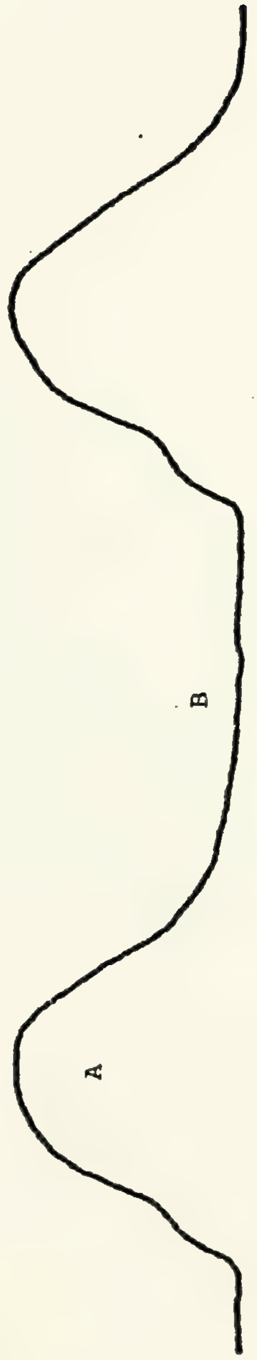

A
B

Figure 4. Double sucrose gap recordings of spontaneous spikes in isolated cat circular muscle. Upper record: slow spikes; lower record: fast spikes. Arrows indicate prepotentials. (Courtesy of Dr. R. Weigel)

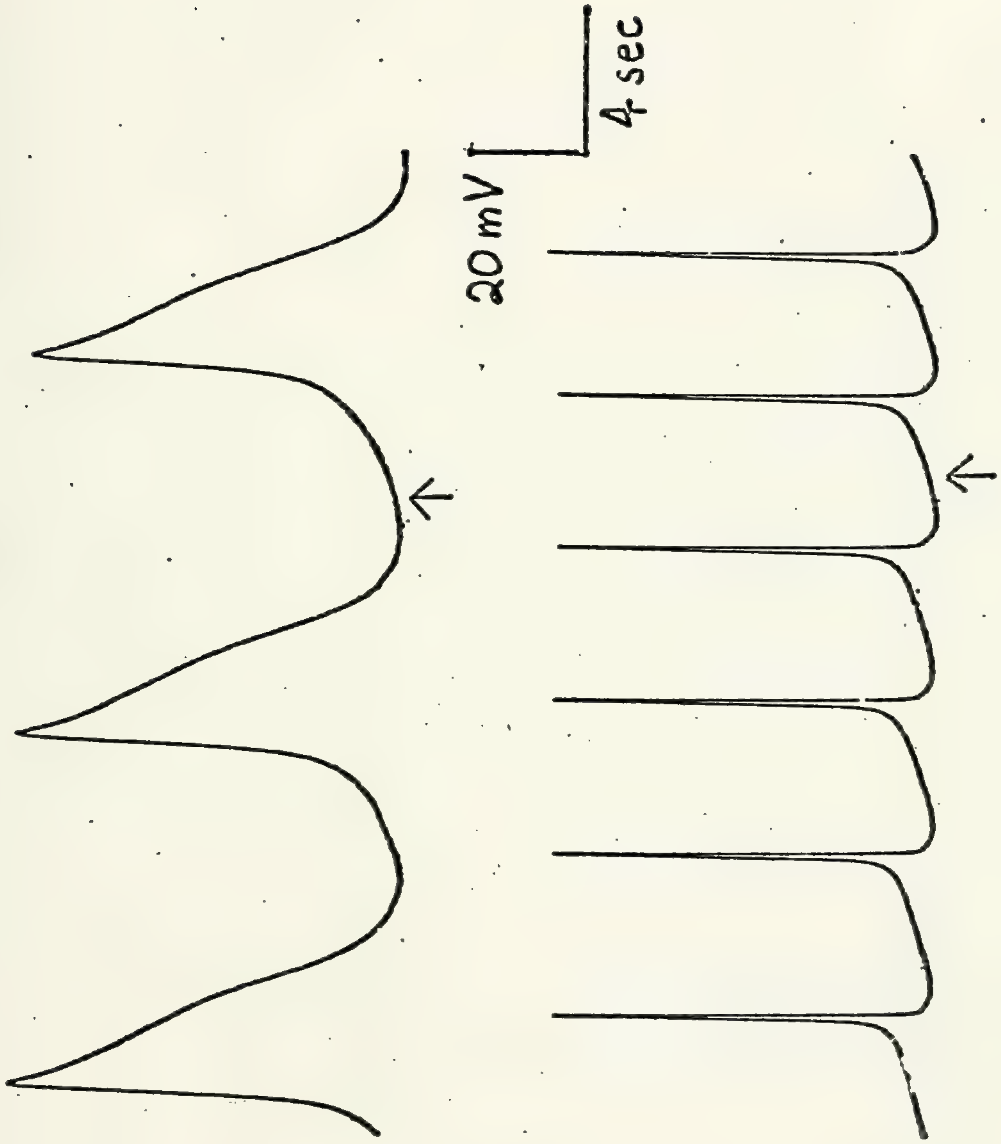
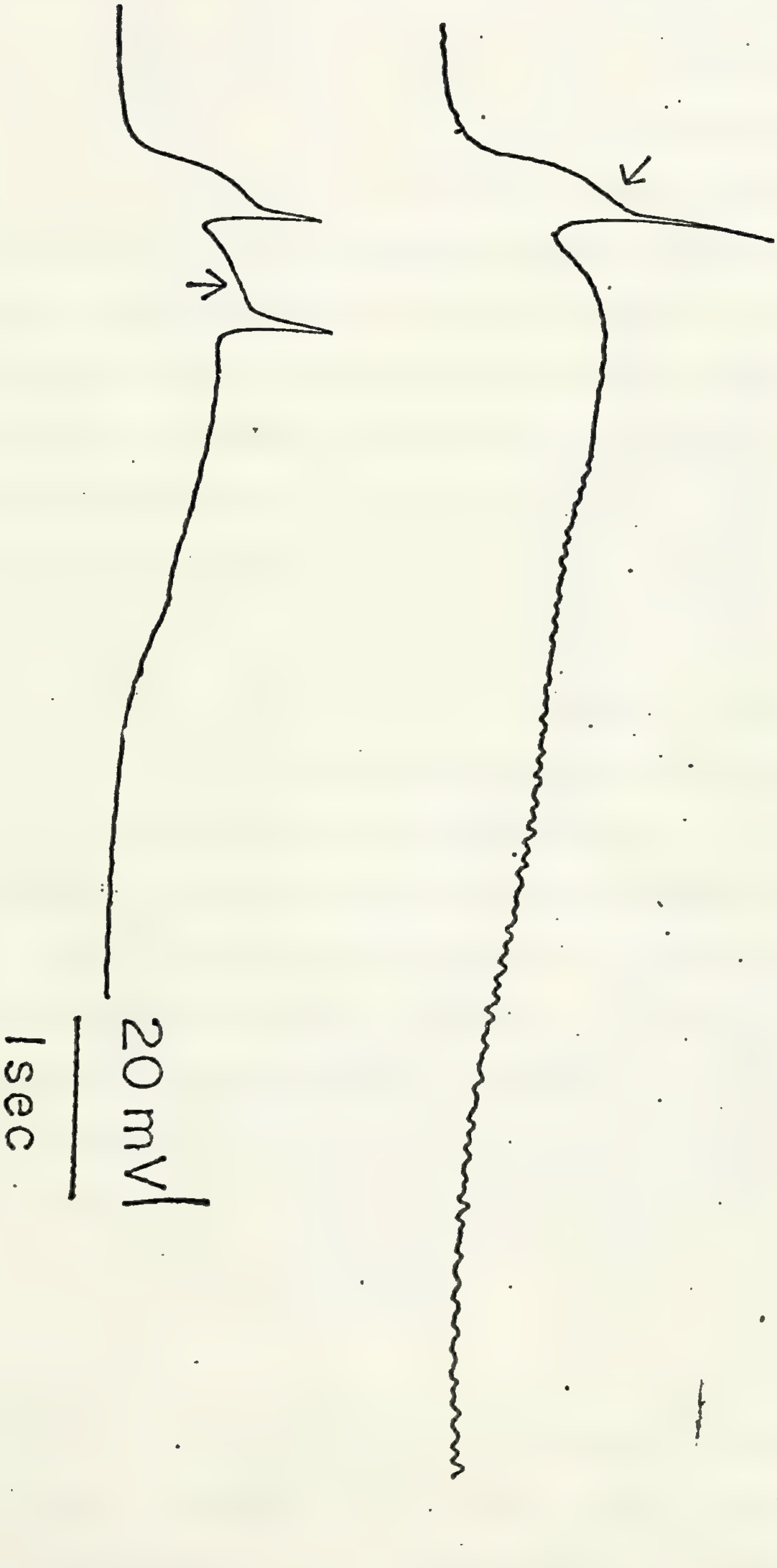

20 mV
4 sec

Figure 5. Intracellular recordings from intact cat intestine showing the relationship of slow waves, prepotentials (arrow) and spikes. Upper record is recorded from longitudinal muscle; lower record is from circular muscle in which the electrode was inserted through a 1 mm slit in the longitudinal muscle layer. Recordings are from different preparations.


20 mV
1 sec

cat small intestine and rat uterus are usually preceded by slowly rising pacemaker potentials (prepotentials) similar to those observed in cardiac muscle (Figure 4) (34,40,41,42,81,155,160,178,247,289). If prepotentials do not reach threshold, spikes do not occur. Often apparent pacemakers do not lead into successful spike discharge and the aborted pacemakers are irregularly mixed in with pacemakers and accompanying spikes (40,42,81,160, 236). In cat small intestine, prepotentials are 1-5 sec in duration, up to 20 mV in amplitude and are present in both longitudinal and circular muscle layers (81,178,236). Application of ouabain or removal of external sodium or potassium does not eliminate prepotentials in either taenia coli or small intestine, but reduction in Ca_o^{2+} or application of Ca^{2+} channel blockers does eliminate the prepotentials (81,178,247,248). Spontaneous prepotentials may result from time and voltage dependent increases in Ca^{2+} conductance (41,178,247, 248). Prepotentials have also been recorded from intestinal muscle of several other species and from bladder (83) and ureter (168).

Recordings from intact or isolated longitudinal muscle show prepotentials superimposed on the rising phase of the slow wave. The slow wave depolarization is thought to activate the increase in Ca^{2+} conductance responsible for initiation of the prepotential (122,178). Prepotential repolarization is probably due to an increase in potassium conductance. The relationship of prepotentials to spikes and to slow waves in cat small intestinal muscle is illustrated in Figure 5.

D. Spikes

Spikes are considered to be the trigger for contractions in smooth muscle (33,40,178,231,233). Many smooth muscle spikes result from an increase in calcium conductance and it is not clear whether an influx of Ca^{2+}

associated with the spike (231), release of internal Ca^{2+} due to the spike depolarization (36,141,187), or both, are involved in triggering contractile activity. Evidence for intracellular Ca^{2+} stores which can support contractile activity without an influx of Ca^{2+} from the bathing solution, has been presented (36,187).

Spikes are present in isolated longitudinal or circular muscle as well as in intact preparations (80,81). In intact cat intestinal muscle cells, which have resting potentials of approximately -60 mV, spikes seldom exceed -20 mV (80), but in taenia coli or isolated cat circular muscle they can overshoot 0 mV (42,80). Spikes are generally between 40-220 msec in duration.

Removal of all but 20 mM of external Na^+ (Na_o^+) has little effect on spikes recorded from guinea pig taenia coli (43,138,139,168,230). The Na^+ channel blocker TTX also has no effect. Further reduction of Na_o^+ (to 2-16 mM), by either sucrose or Tris replacement, results in increased amplitude and rate of rise of spikes (37,43,168). Following reduction in Na_o^+ levels, the threshold for spike generation usually increases without accompanying membrane potential change. After prolonged exposure to low Na^+ saline, spikes often deteriorate.

Removal of Ca_o^{2+} results in elimination of spikes recorded from guinea pig taenia coli (43,168,287). The effects of diminished Ca^{2+} are greater in saline with high Na^+ levels. Spike amplitude and rate of rise decrease in saline low in Ca^{2+} and are increased following increases in Ca_o^{2+}. These observations suggest that spikes result from increases in Ca^{2+} conductance but an antagonism between Na^+ and Ca^{2+}, as demonstrated by increased spike height following reduction in Na_o^+, may exist.

Further support for a Ca^{2+} mediated spike in taenia coli comes from elimination of spikes by Ca^{2+} channel blockers (Mn^{2+}, Co^{2+}, verapamil)

(44,119,130,165,230). Other divalent cations, Sr^{2+} and Ba^{2+}, can substitute for Ca^{2+} as an inward current carrier and can support spikes (43,168,230). Following addition of Ba^{2+} to Krebs solution with or without Ca^{2+} the spike duration is extended and the rate of repolarization decreased (43,287). This has been attributed to the inability of Ba^{2+} to activate a K^+ conductance normally activated by Ca^{2+} (198). Thus, in guinea pig taenia coli spike depolarization results from an increased Ca^{2+} conductance, and repolarization results from an increase in K^+ conductance.

Spikes are also present in both muscle layers of cat small intestine and may result from an increase in Ca^{2+} conductance. Following reduction in Ca_o^{2+}, spike amplitude and rate of rise decrease (43,75,178,230); increases in Ca_o^{2+} result in increased spike amplitude. Spikes are eliminated by Ca^{2+} channel blockers but are unaffected by TTX (178,230,301). Spike amplitude is relatively insensitive to reduction in Ca_o^{2+} over a 2-3 fold range as long as the ratio of $\sqrt{Ca^{2+}}/Na_o^+$ remains constant (75).

Substitution of Ba^{2+} or Sr^{2+} for Ca^{2+} permits spike production in circular muscle of cat small intestine (230,301). As in guinea pig taenia coli, Ba^{2+} does not activate the outward transient current, and spikes of prolonged duration occur. Similar results are observed following TEA, a K^+ channel blocker (80,301). In cat small intestinal circular muscle the initial inward current of the spike is Ca^{2+} mediated, and the influx of Ca^{2+} activates a K^+ current which produces membrane repolarization (301). The entry of Ca^{2+} may be antagonized by Na_o^+.

The circular muscle of cat small intestine generates a second type of spike, "slow-spikes" (Figure 4). These "slow-spikes" are of similar magnitude to the more commonly observed spikes but are 1-5 sec in duration

(80). A voltage and time dependent increase in Ca^{2+} conductance appears to be responsible for the depolarizing phase of the slow-spikes. After TEA application fast spikes are converted into slow-spikes. Hence, it has been suggested that slow-spikes occur in cells when K^+ conductance is only slightly activated (80).

Spikes are often superimposed on the plateau of stomach action potentials. In guinea pig stomach, spikes are sensitive to removal of Ca_o^{2+} or to application of Ca^{2+} channel blockers (147,166). Spike amplitude is enhanced and rate of fall decreased by TEA (147). After substitution of Sr^{2+} for Ca^{2+}, spikes are still produced (147). Application of TTX or atropine, or removal of Na_o^+, does not eliminate spikes (166). Spikes recorded from the stomach of cat and dog have also been found to be sensitive to removal of Ca_o^{2+}.

In rat portal vein following TEA treatment, all or none Ca^{2+}-dependent spikes occur (86). Strontium can substitute for Ca^{2+} as an inward current carrier, In contrast to portal vein spikes, spikes recorded from sheep arterial muscle are more dependent on Na^+ than on Ca^{2+}. Keatinge found that sheep common carotid artery was electrically quiescent in normal saline, but 20-80 min after removal of Ca_o^{2+}, spontaneous spike discharge occurred (156). These spikes were eliminated by Na_o^+ replacement. In saline with normal Ca^{2+} levels, but Na^+-free, small spikes could be induced by membrane depolarization. Application of TTX did not eliminate either spontaneous or induced spikes. In a subsequent study Keatinge (157) measured an increased influx of $^{24}Na^+$ during electrical activity induced by Ca^{2+} deprivation. He concluded that Na^+ is the predominant current carrier of the depolarizing phase of carotid artery spikes.

Spikes induced in guinea pig superior mesenteric artery by TEA are sensitive to alterations in Ca^{+}_{o} or addition of verapamil but insensitive to changes in Na^{+}_{o} or application of TTX. The inward current of the TEA-induced spikes is carried by Ca^{2+} which enters through TTX insensitive, verapamil sensitive, channels (129).

Although uterine muscle has been extensively studied with respect to the ionic mechanism of spike generation, there is not complete agreement on a single mechanism of generation. Anderson et al. in studies on spikes in rat uterine smooth muscle, demonstrated that after reduction of either Na^{+}_{o} or Ca^{2+}_{o} the initial inward current was reduced and that neither Na^{+} nor Ca^{2+} alone was capable of supporting spikes (10). Spikes were eliminated by Mn^{2+} but unaffected by TTX. They concluded that spike generation in uterine muscle may involve a single transient conductance channel and activation of the channel appears to require both Na^{+} and Ca^{2+}.

Kao and McCullough also analyzed the ionic currents responsible for uterine muscle spikes (155). They demonstrated both Ca^{2+} and Na^{+} dependency for the spike upstroke. An outward current, due to K^{+}, was responsible for spike repolarization. It was concluded that Na^{+} is the predominant current carrier in the depolarizing phase of the spike, while Ca^{2+} may act to regulate Na^{+} entry. Spike repolarization was found to be due to an increase in K^{+} conductance. In contrast, Mirroneau found Ca^{2+} to be the predominant current carrier in the spike depolarizing phase with only a minor role attributable to Na^{+} (205).

One possible explanation for the different roles attributed to Na^{+} and Ca^{2+} in uterine muscle spike generation is hormonal variation in the tissues. Anderson et al. used estrogen-treated ovariectomized rats, Kao and McCullough

used rats after 3 weeks of pregnancy and Mirroneau rats 18 days after gestation. What role differing endocrine environments play in generation of electrical activity is not known.

In guinea pig and mouse myometrium, large amplitude spikes can be elicited in Na^+ free saline for over 15 min (90,220,221,279). The rate of fall of guinea pig myometrial spikes is decreased by TEA, suggesting a role of K^+ in spike repolarization (279).

In the longitudinal muscle layer of the stomach of toad and skate, spontaneous slow-spikes have been recorded (188,231). The slow-spikes are 4-20 sec in duration and are not eliminated by ouabain application or removal of Na^+_o. Slow-spike amplitude and frequency are sensitive to reduction in Ca^{2+}_o and to application of Ca^{2+} channel blockers. The stomach slow-spikes of toad and skate may result from increases in Ca^{2+} conductance (188).

E. Action Potentials in Stomach and Ureter

Action potentials recorded from stomach are different from intestinal slow waves. In cat (224), guinea pig (216,217) and dog (108) stomach, action potentials consist of two components, an initial rapid depolarization and a plateau potential. Gastric action potentials vary considerably in the different regions of the stomach. El-Sharkaway *et al*. (108) showed the corpus area of dog stomach to have action potentials of frequency 3.7 c/min and amplitudes of 28.5 mV, while the pyloric ring action potentials had frequency of .15 c/min with 71.3 mV amplitudes. Intermediate regions had activity of intermediate frequency and amplitude. Detailed analyses of the different regions of cat and guinea pig stomach have not been made. In cat stomach, action potentials originate in the longitudinal muscle layer (224) but in guinea pig stomach they can be generated by the circular muscle layer (216,217). Action potentials are present in isolated longitudinal muscle of dog stomach (280).

The initial rapid component of cat stomach action potentials is sensitive to Na^+ removal or ouabain application (224). The slower component is Ca^{2+} sensitive (224). Neither component is eliminated by TTX but the second component is sensitive to Ca^{2+}-channel blockers. The frequency of action potentials is decreased by reduction in Ca_o^{2+} and enhanced by high Ca_o^{2+}. These effects of Ca_o^{2+} on action potential frequency require Na^+ in the bathing medium. It was concluded that the first component of the action potential represents a Na^+ dependent process similar to that in intestinal slow waves. The second component may reflect a Ca^{2+} conductance change similar to that in spikes.

Action potentials in guinea pig stomach are also composed of two components (216,217). The slower small (10-15 mV) component is insensitive to membrane potential changes and may reflect an unknown metabolic process regulated primarily by Ca^{2+} and to a lesser extent by Na^+ and K^+. It was concluded that the metabolic process is not an oscillatory Na^+ pump. The second component of the action potential is generally larger than 20 mV and supports the plateau region. This component is due to a voltage-dependent Ca^{2+} conductance change.

The ionic basis of the plateau potential of canine gastric action potentials has been investigated (108). In saline with low Ca^{2+} or Na^+; or after D600 application, the plateau potential becomes reduced. It was concluded that both Ca^{2+} and Na^+ take part in generation of the plateau potential.

Action potentials of the ureter are also composed of several components. The action potentials consist of an initial rapid depolarization, which

decays into a plateau phase with superimposed small spikes. In guinea pig, reduction in Na_o^+ below 16 mM increases the amplitude of the initial depolarization, while decrease in Ca_o^{2+} eliminates the initial depolarization and spikes (168,270). Strontium can substitute for Ca^{2+} as an inward current carrier but prolongs the plateau phase as does TEA (270). Action potentials are not affected by TTX (167).

In contrast to the initial depolarization and spikes the plateau phase is eliminated by removal of Na_o^+ (168,270). Spontaneous spikes do not occur in Na^+ free saline but they can be elicited by membrane depolarization. Thus, in guinea pig ureter the initial rapid depolarization and spikes are due to an increased Ca^{2+} conductance followed by an increase in K^+ conductance. The plateau phase is Na^+ dependent (167,168,270).

Kobayashi (161) found that high Ca_o^{2+} increased the amplitude and rate of rise of the initial phase of cat ureter action potentials. Removal of Na_o^+ abolished action potentials but they reappeared if Ca_o^{2+} was increased. Kobayashi (161) concluded that in normal saline, Na^+ is the predominant inward current carrier of the action potential but that in high Ca^{2+} saline, Ca^{2+} may enter as the principal current carrier.

F. ACh Induced Activity

Spontaneous slow oscillations have been recorded in intact segments of guinea pig intestine (27,225). The oscillations are sensitive to TTX and to atropine and may result from spontaneous liberation of ACh from the myenteric plexus of guinea pig intestine. The slow potentials are eliminated in hypertonic saline (27). Following ACh addition to the hypertonic solution, slow potential oscillations with a period of 1-10 sec develop. These slow potentials are ouabain and TTX insensitive but are sensitive to removal of Na_o^+. Bolton (27) postulated that these slow potentials in guinea pig intestinal muscle result from a voltage dependent increase in Na^+ conductance.

As described later, cat small intestinal "slow waves" are not eliminated by hypertonic saline, but are abolished by ouabain (178,231). Following ACh application, after ouabain, slow oscillations in membrane potential appear (231).

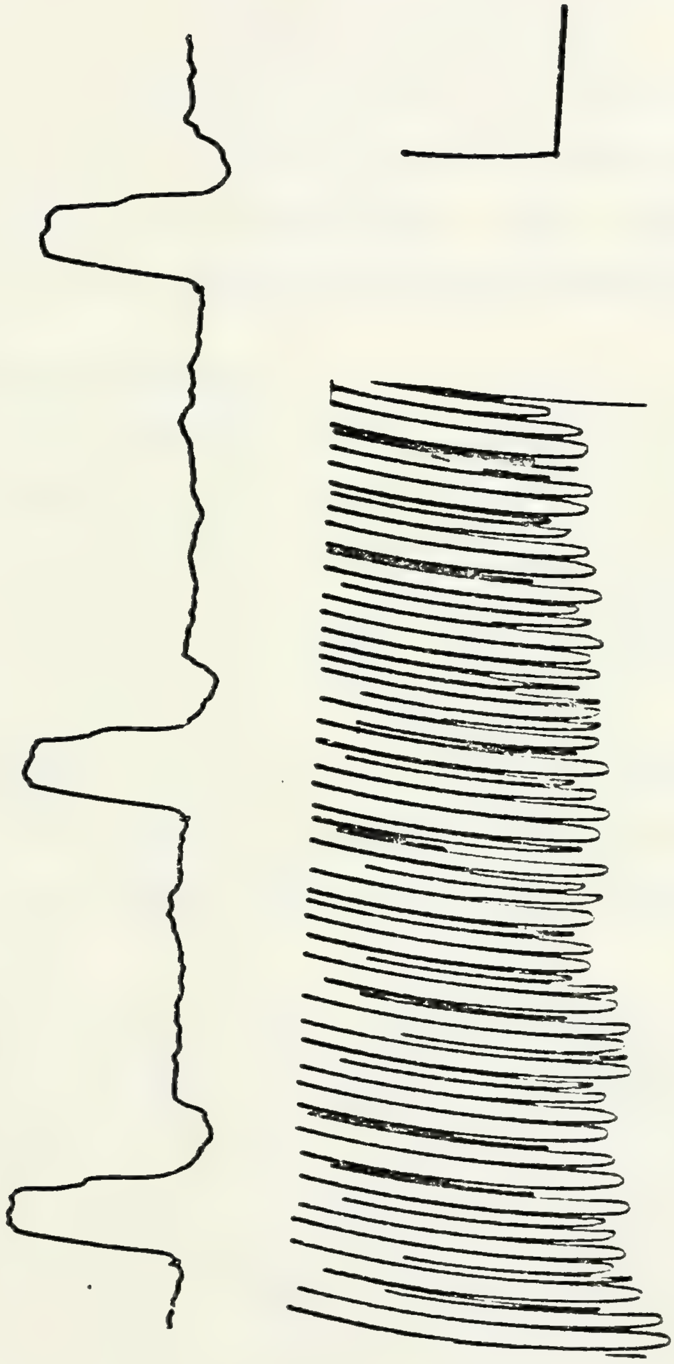
The smooth muscle portion of the esophagus is normally electrically quiescent (71,213). Application of ACh induces slow oscillatory activity (71,213). The slow oscillations are insensitive to ouabain or TTX but are eliminated by removal of Na_o^+ (187).

G. EDTA Induced Activity

Addition of 1-10 mM EGTA to solutions with no added Ca^{2+} results in elimination of spontaneous slow waves and spikes in a variety of smooth muscle preparations (234). Following periods of electrical quiescence, ranging from 5 to 45 min, spontaneous rhythmic depolarizations (prolonged potentials) develop (Figure 6). Prolonged potentials have been observed in cat intestinal muscle and stomach of frog, toad and skate. Prolonged potentials have a mean duration of 17.6 sec, ranging from 4-44 sec. Measured intracellularly, prolonged potentials can be as large as 40 mV.

Prolonged potentials are insensitive to ouabain or TTX application as well as to K^+ or Cl^- removal. In contrast, reduction in Na_o^+ or application of Ca^{2+} channel blockers results in elimination of prolonged potentials. It was concluded that prolonged potentials result from Na^+ ions traversing channels usually used by Ca^{2+}. Pacemaker potentials do not precede prolonged potentials and it is not clear what causes the rhythmicity.

Figure 6. Effects of EGTA on intestinal smooth muscle. Upper trace: prolonged potentials recorded from cat small intestine following treatment with 5 mM EGTA (cal bar: .5 mV, 5 sec). Lower trace: fast potentials recorded from rat small intestine following treatment with .5 mM EGTA (cal bar: .3 mV, 3 sec). Recordings were made with pressure electrodes.



Addition of .3-.7 mM EGTA to Ca^{2+}-free saline induces fast rhythmic potentials in rat small intestine (Figure 6) (186). Fast potentials are eliminated by removal of Na^+_o or following Ca^{2+} channel blockers, and may result from a mechanism similar to that which evokes prolonged potentials. Fast potentials were not observed in cat, mouse, guinea pig, hamster or toad intestine and may be a property of rat small intestine only.

H. Ionic Basis of Slow Waves

Several ionic mechanisms have been proposed to account for slow wave generation in small intestinal smooth muscle. Daniel (87) with dog small intestine; Liu, Prosser and Job (178) and Connor, Prosser and Weems (81) with cat small intestine, hypothesized that slow waves result from oscillations in the activity of an electrogenic Na^+ pump. Slow waves are eliminated by ouabain application or by removal of K^+_o, treatments which inhibit Na^+ pump activity. The initial effect of ouabain is on slow wave amplitude. Subsequently there are small changes in slow wave waveform and frequency (81,151,232).

According to this hypothesis, when the electrogenic Na transport system is operating at a low level of activity, the membrane potential is depolarizing. Following increases in the transport rate, the membrane potential hyperpolarizes. The diastolic phase of the slow wave corresponds to periods of high levels of electrogenic pump activity (with respect to the systolic phase). Changes in the waveform of slow waves can, therefore, be interpreted as changes in the level of electrogenic Na transport.

Additional support for the oscillating Na^+ pump hypothesis came from studies in which Job demonstrated Na^+ efflux to be maximal during slow wave repolarization and influx maximal during depolarization (151). Slow waves

were found to be sensitive to metabolic inhibitors (152). Job postulated that oscillatory ATP levels may cause oscillations in the membrane potential (152). In his scheme a build-up of ATP at the internal membrane surface results in increased Na^+ conductance and therefore the membrane potential depolarizes. The resultant increase in internal Na^+, concomitant with high levels of ATP near the membrane, stimulates the Na^+ pump and the membrane potential repolarizes.

Connor, Prosser and Weems (81) provided substantial support for the oscillating Na^+ pump hypothesis. Strips of isolated longitudinal muscle from cat small intestine were examined in the double sucrose gap. Application of ouabain or incubation in K^+-free solution eliminated slow waves with the membrane potential depolarizing to the slow wave peak. Repolarization of the membrane potential did not restore slow waves. Depolarization of the membrane potential to the peak of the slow wave, by treatments which eliminate Na^+ pump activity, supports the hypothesis that slow waves result from electrogenic Na^+ pump activity. Additional support for this hypothesis came from voltage clamp studies. Voltage clamp of the membrane at the resting potential produced spontaneous inward-directed current transients (81). The current transients were at the same frequency as slow waves and were similarly sensitive to ouabain. From membrane currents measured under voltage clamp and from passive membrane resistances and capacitances measured under current clamp, slow waves were reconstructed. Recorded slow waves agreed well with reconstructed ones, which were based on the assumption of a cyclic electrogenic Na^+ pump.

El-Sharkaway and Daniel proposed that rabbit small intestinal slow waves do not result from oscillatory electrogenic Na^+ pump activity but

from sequential conductance changes first to Na^+ and then to Cl^- (106,107). According to this hypothesis, the initial upstroke of the slow wave is supported by an increase in Na^+ conductance. The membrane potential depolarization is sustained by an increase in Cl^- conductance. In these studies incubation in K^+-free saline or in ouabain-containing solutions resulted in elimination of slow waves, but the membrane potential remained 8-15 mV more hyperpolarized than the slow wave peak. After anion substitution for Cl^-, decreases in slow wave duration and frequency were observed. In saline containing 10% of normal Na^+, slow waves were abolished, but in solutions containing 50% of normal Na^+ levels, slow waves were not affected.

The apparent experimental discrepancies between these studies (106,107) and the previous ones (81,151) can be partially explained by the different nature of the preparations used. Connor, Prosser and Weems (81) used strips of isolated longitudinal muscle while El-Sharkaway and Daniel used preparations highly contaminated with circular muscle (see method ref. 107). If the circular muscle acts as an amplifier of longitudinal muscle activity (32), then elimination of slow waves recorded from preparations containing circular muscle would result in depolarization of the membrane to the peak of the isolated longitudinal muscle slow wave, not to the peak of the amplified slow wave. Hence, the membrane potential would settle to a level more hyperpolarized than the slow wave peak.

Subsequent to the studies by El-Sharkaway and Daniel (106,107), Christofferson (72) demonstrated chelation of Ca^{2+} by the anions they used to substitute for Cl^-; the effects of reduction in Cl^-_o in rabbit intestine are identical to reduction in Ca^{2+}_o in cat intestine (80,164). This may be a likely explanation for the effects of reduction in Cl^-_o. The failure of removal of 50% of Na^+_o to affect slow wave amplitude or rate of rise is not

indicative of a Na^+ conductance change producing slow waves. In other preparations (136) where a Na^+ conductance change is involved in generation of electrical activity (e.g., Na spikes), reduction in external Na^+ results in decreased amplitude and rate of rise of potential changes as predicted by a Nernst relationship.

Slow Waves in Other Preparations

Slow waves recorded from guinea pig taenia coli have an average amplitude of 13 mV and duration of 0.9 sec (43). In high Na^+ saline, slow wave duration is prolonged and amplitude increased (43). In low Na^+ saline, slow waves have decreased amplitude and increased duration. In Na^+ free saline, slow waves are eliminated and in Ca^{2+} free saline, slow wave frequency increases and amplitude decreases.

Tomita and Watanabe (288) proposed a mechanism to account for slow wave generation in guinea pig taenia coli, uterine and ovarian smooth muscle. According to their scheme K^+ conductance is mediated by intracellular Ca^{2+} levels. A metabolically dependent Ca^{2+} transport system regulates the intracellular Ca^{2+} level. When intracellular Ca^{2+} is high so is K^+ conductance and the membrane hyperpolarizes. Increasing the Ca^{2+} transport rate, i.e., reducing Ca_i^{2+}, results in a decrease in K^+ conductance and the membrane depolarizes. Cyclic activity of the Ca^{2+} transport system may result in oscillatory membrane potential. According to this scheme membrane hyperpolarization results from an increase in K^+ conductance, while membrane depolarization results from an influx of Na^+ into the cell during a time when the potassium conductance is low. The Tomita and Watanabe hypothesis seems not to explain cat small intestinal slow waves. According to this hypothesis, decreases in Ca_o^{2+} would produce a decrease in K^+ conductance.

A decrease in K^+ conductance would result in shortening of the diastolic phase of the slow wave. This is in contrast to the experimental observations that reduction of Ca_o^{2+} causes a prolongation of the diastolic phase of cat small intestinal slow waves (80).

Slow waves have also been recorded from cat colon (70). These slow waves have duration 3-19 sec and amplitude 12 mV. In contrast to small intestinal slow waves, colonic slow waves are generated by the circular muscle layer of the colon. Slow waves are not present in the caecum (11). Weinbeck and Christiansen (303) altered the ionic composition of the solution that bathed colonic muscle strips. They concluded that these slow waves are dependent on the presence of both Na^+ and Ca^{2+}.

(ii) Regulation of Ca_i^{2+}

The concentration of Ca^{2+} in the cytoplasm of cells is low (10^{-8} - 10^{-7} M) with respect to the extracellular Ca^{2+} level (10^{-3} M). Intracellular Ca^{2+} must be maintained at this low level for proper regulation of various intracellular events by Ca_i^{2+} (see Table 1) (ref. 56). Several specific transport systems have been shown to be involved in maintaining the level of Ca_i^{2+}: Ca^{2+} extrusion by the plasma membrane; Ca^{2+} accumulation by sarcoplasmic and endoplasmic reticulum; and Ca^{2+} uptake by mitochondria.

A. Plasma Membrane Extrusion

(1) CaATPase

In 1966 Schatzman demonstrated that the rate of ^{45}Ca efflux from red cell ghosts or intact red cells, which had been Ca loaded, was larger than the rate of inward Ca movement (258,262). This efflux required intracellular ATP and Mg^{2+}. ADP, AMP, pyrophosphate or acetylphosphate could not support the active Ca extrusion (262,263,264). GTP, ITP, CTP or UTP could substitute for ATP when incorporated into resealed ghosts although the usual energy source for the transport system was ATP (174,219). The proportion of total ATP required for Ca extrusion is much less than that required for Na extrusion by the Na-K pump (174). In red blood cell ghosts or intact cells, ATP levels must be reduced to less than 10% of normal before active Ca transport is abolished (174). A complex of Mg^{2+} and ATP (MgATP) rather than ATP, may be the active substrate of the CaATPase (304). The K_m value for MgATP is approximately 50 μM. The K_m for Ca_i^{2+} is between .1-4 μM and the enzyme has a V_{max} value of 85-200 μM Ca transported per liter of cells per minute (at 37°C) (249,257,263,265). The molar ratio of Ca^{2+} transported,

Table 1. Ca^{2+} dependent reactions in cells. From Carafoli and Crompton, ref. 56.

Table 1

- Activation of enzyme systems
  - Glycogenolysis (phosphorylase-b kinase)
  - Lipases and phospholipases
  - α-Glycerophosphate dehydrogenase
  - Pyruvate dehydrogenase
  - Succinate oxidation
  - Synthesis of some phospholipids
  - NADH dehydrogenase (plant mitochondria)
  - Interaction of cytochrome c with the mitochondrial membrane
  - Light emission
  - Decision to divide

- Inhibition of enzyme systems
  - Pyruvate kinase
  - Synthesis of some phospholipids
  - Substrate oxidation (NADH leakage) in lung mitochondria

- Activation of contractile and motile systems
  - Muscle myofibrils
  - Cilia and flagella
  - Microtubules and microfilaments
  - Cytoplasmic streaming
  - Pseudopod formation

- Hormonal regulation
  - Formation and/or function of cAMP (GH, LH, TSH, MSH, PTH)
  - Release of insulin, steroids, vasopressin, oxytocin, catecholamines, thyroxine, and progesterone

- Membrane-linked functions
  - Excitation-secretion coupling at nerve endings
  - Excitation-contraction coupling in muscles
  - Exocrine secretion (pancreas, salivary glands, and HCl in stomach)
  - Aggregation of platelets
  - Action potential (nerve and muscle cells)
  - Na^+, K^+-ATPase of several membranes
  - Tight junctions
  - Cell contact
  - Binding of prostaglandins to membranes

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per ATP split, has been reported to be between 1 and 2, although most recent studies give a value of 2 Ca^{2+} transported per ATP hydrolyzed (116,238,258).

Active Ca^{2+} extrusion in human erythrocytes is independent of Ca_o^{2+}. Addition of EGTA to eliminate Ca^{2+} from the bathing media does not alter the rate of Ca efflux from that obtained with 2-5 mM Ca_o^{2+} (174,249,257, 263,271). Increasing Ca_i^{2+} produces a maximum rate of active Ca efflux of 4-10 mM/l cells hr (249,257,263,265,289).

The active extrusion of Ca in red cells is not inhibited by ouabain or oligomycin or dependent upon a transmembrane gradient of Na^+ or K^+ (174, 257,267). Mg^{2+}, Mn^{2+} or Cu^{2+}, when introduced into resealed ghosts, are not transported by the Ca transport system, while Sr^{2+} can compete with Ca^{2+} for transport sites (219,257,267). The active Ca transport system has a Q_{10} of 3.5-3.8 and Cha (62) reported rapid inactivation at high temperatures (46-50°C). The system has a pH optimum between 7.5-8.0 (174, 257,265).

Tetracaine, chlorpromazine and propranolol inhibit active Ca transport. Schatzman (264) has suggested that the probable mechanism is destruction of membrane stability. Several sulfhydryl reagents such as mersalyl, ethacrynic acid and PCMBS also result in marked inhibition of Ca extrusion (257,267). The action of these drugs is complicated by an accompanying increase in Ca^{2+} influx. Ruthenium red, an inhibitor of mitochondrial Ca uptake, partly inhibits active Ca extrusion in red cells (264). The most effective inhibitors of active Ca extrusion in red blood cells are trivalent lanthanum cations, lanthanides and possibly vanadate (100,266). Holmium or praseodymium at 1 mM levels block red cell active extrusion. Sarkadi et al. (257) reported a 95% inhibition of active Ca transport by .1-.2 mM La^{3+}.

Recent studies on squid giant axon have presented evidence that Ca extrusion is mediated by an ATP driven Ca pump. Brinley et al. (39) measured Ca efflux over a range of Ca_i^{2+} concentrations from 5-10,000 nM. The results demonstrated a linear dependence of Ca efflux on Ca_i^{2+} in the range of 5-100 nM. Over this range Ca efflux was insensitive to Na_o^+ or Ca_o^{2+} removal. Dipolo and Beague (92,93) observed ATP dependent Ca extrusion in the absence of a Na^{2+} gradient. They observed a portion of the Ca efflux uncoupled to other ionic movements. In these studies it was concluded that under physiological Ca concentrations, CaATPase plays an important role in Ca extrusion from squid axons.

In addition to the Mg dependent CaATPase, another CaATPase, which requires higher internal Ca^{2+} levels but does not require Mg^{2+}, has been reported in red cells (158,250,264). Schatzman (263) suggests that this second ATPase may be an artifact. The presence of a Mg and ATP dependent Ca^{2+} extrusion mechanism has also been reported in: liver (294); kidney (210); L cells (171); neurons (99,100); smooth muscle (58,84,85).

(2) Na-Ca Exchange

In 1969 Blaustein and Hodgkin (25,211) showed that the efflux of Ca from squid axons previously loaded with Ca was sensitive to removal of Na_o^+ or Ca_o^{2+}. Addition of CN^- produced a large increase in Ca efflux which was reduced by decreases in Na_o^+ (13,25). Following return to normal Na_o^+ levels, the Ca efflux increased. These experiments supported the hypothesis developed by Reuter and Seitz (244) for cardiac muscle [a modification of Ussing's hypothesis (290a)] that Ca efflux might use the energy in the Na^+ gradient across the plasma membrane to exchange Ca for Na. The transport ratio of Na:Ca has been postulated to be between 2 and 4 (25,102,211).

Dipolo (97,98) showed that the efflux of Ca from dialyzed axons increased when ATP was added to the perfusion fluid. Baker (14) found that ATP is not hydrolyzed during Ca efflux and concluded that in the presence of ATP the affinity of the transport sites for both Na^+ and Ca^{2+} increases. The K_m of Ca^{2+} for activation of Ca^{2+} efflux is .3-20 μM in the presence of ATP and as high as 2 mM in its absence. The K_m for Na_o^+ is 120 mM in the presence of ATP. The K_m of the system for ATP is approximately 600 μM (14,211). Thus, ATP may have more of a regulatory than activating role in Ca transport.

Na_o^+ activates Ca efflux in a sigmoidal manner. The efflux of Ca is inhibited by increases in Na_i^+ (26,39). This inhibition may result from increases in Na_i^+ decreasing the energy of the Na^+ gradient across the plasma membrane or from a competition of Na_i^+ or Ca_i^{2+} for attachment to the same carrier or site. The Na:Ca exchange system is not inhibited by ouabain, oligomycin or by metabolic inhibitors (13,39,211).

The existence of a coupled Na:Ca exchange has also been postulated in several other systems: skeletal muscle (57); kidney (45); vagus nerve (154); cardiac muscle (244); smooth muscle (24,180).

B. Sarcoplasmic and Endoplasmic Reticulum

Most of the studies of Ca^{2+} transport by sarcoplasmic reticulum have been done on membrane fragments which are produced by disruption of the sarcoplasmic reticulum during whole tissue homogenization (145,181,191). Membrane fragments reseal into closed vesicles. Ca ions are accumulated by the vesicles during an energy dependent process. Approximately 2 Ca ions are accumulated by the vesicles per ATP hydrolyzed (103,131,133,300). The binding of ATP to the Ca transporting system requires Mg^{2+} (103) and a

MgATP complex may be the active substrate with a K_m of approximately 10 μM (68,144,145,201). Unlike plasma membrane CaATPase (258), reversal of the Ca^{2+} gradient can result in ATP production by the sarcoplasmic reticulum CaATPase (185). The k_m for Ca ion uptake by the vesicles is approximately 1 μM (range .03-10 μM) with a V_{max} of .4-3.6 μmoles Ca/min/mg protein and maximum uptake of .14-6 μmoles/mg protein (55,132,133).

High Ca^{2+} levels within the sarcoplasmic reticulum inhibit CaATPase activity (131). Found in high levels within the sarcoplasmic reticulum are 2 proteins which bind Ca^{2+} (145,200), thus, alleviating the possibility of inhibition of CaATPase by Ca^{2+}. Calsequestrin, a water soluble acidic protein, binds 43 moles of Ca per mole. Ca binding protein (CBP) binds Ca^{2+} at either high (K_d = 3 μM) or low (K_d = 100 μM) affinity sites (69).

Ca accumulation by cardiac and slow skeletal muscle sarcoplasmic reticulum is similar to the process in fast skeletal muscle except the rate of extent of Ca uptake is less (55,109,191,273). The V_{max} for cardiac and slow skeletal muscle ranges from .1-.15 μmoles Ca/min/mg protein with a maximum uptake of .04-1.2 μmoles/mg protein (55).

The endoplasmic reticulum of liver (55,209), kidney (55,210), brain (55,93,222,223), salivary glands (6,55) and platelets (55) also transport Ca in an energy dependent fashion. All the systems are energized by ATP and require Mg. The K_m for Ca transport ranges from 4.6 in liver to 100 μM in salivary gland (6,55,209). Maximum capacity for Ca uptake is between 50 nmoles/mg protein in kidney and brain to 2750 nmoles/mg protein in salivary gland (6,55,209,210,222,223). In liver maximum velocity of Ca uptake is .1 nmole Ca/sec (55,209).

C. Mitochondria

Mitochondria isolated from nearly all sources, with the exception of yeast mitochondrion (53), accumulate Ca^{2+} (49,55,67,92,175,260,274,295). Ca^{2+} can be accumulated by energy independent or dependent processes. The energy independent accumulation was described first by Slater and Cleland (272) and subsequently by several investigators. In the absence of respiration and ATP, mitochondria bind 30 nmol Ca/mg protein with a K_m of 100 μM. This type of Ca binding is nonspecific and monovalent and divalent cations, as well as local anesthetics can compete for the same site (65,252,259). In addition to these low affinity sites, high affinity Ca sites are also present in nonenergized mitochondria. These sites bind .1-1 nmole Ca/mg protein with a K_m of .025 μM (245,256).

Energy dependent Ca^{2+} uptake by mitochondria can be energized by ATP hydrolysis or coupled substrate oxidation. 100 nmol Ca/mg protein can be accumulated but in the presence of acetate or phosphate can increase to 500 nmol/mg protein. For each pair of electrons passing from NADH to oxygen, approximately 6 Ca ions are accumulated (1.6-2 Ca ions are accumulated for each pair of electrons traversing each phosphorylating site) (1, 64,176,253).

The energy dependent Ca uptake is inhibited by lanthanides or ruthenium red, which at low concentrations do not inhibit respiration or transport of other ions (208). The binding of lanthanides to the Ca transport system occurs with a high affinity ($K_d = 10^{-8}$ M). Ca transport by mitochondria can also be inhibited by 3 other types of inhibitors: respiratory inhibitors (rotenone, CN^-, antimycin A) (111,112,271); ATPase inhibitors (oligomycin) (172); and uncouplers (DNP) (134,135). Since the inhibition

of respiration can be bypassed by ATP hydrolysis, complete inhibition of energy dependent Ca transport requires both inhibitors of respiration and of ATPase (258).

There is considerable disagreement in the literature on the kinetics of energy dependent Ca^{2+} uptake by mitochondria. The reported K_m values for Ca^{2+} transport varies from .1-70 μM (50,51,52,73,149,241,276,295). The differences in K_m values can not be attributed to differences in tissue type but may partially be accounted for by different types of experimental techniques used in the determinations. Lower K_m values are obtained when Ca^+ uptake is estimated by measurement of ^{45}Ca distribution in mitochondria and in the medium, or when calculated from O_2 uptake or H^+ release experiments, than when kinetic measurements of mitochondrial and medium Ca^{2+} levels are determined by Ca electrodes and Ca sensitive dyes (258). A sigmoidal relationship exists between Ca_o^{2+} and rate of Ca uptake (50,143,261, 297). The binding of 2 Ca ions to the transport system may be required for Ca to be transported (50,143,297).

The V_{max} of energy dependent Ca uptake ranges from 2-12 nmole Ca/sec mg protein (261,297). The highest values are obtained when Ca uptake is measured a few hundred milliseconds after Ca^{2+} addition. Intermediate values (4-5 nM/sec mg protein) are obtained when Ca uptake is measured a few seconds after Ca^{2+} addition and the slowest rates result from measurements made 10 or more seconds after Ca^{2+} addition.

Mg^{2+} competes with Ca^{2+} for the transport system when its concentration is several orders of magnitude higher than Ca^{2+} (149,176,276). Hence measurements made in the presence of Mg^{2+} may not reflect the true affinity of the system for Ca^{2+} but may represent a more physiological condition (258). Sr^{2+} and Ba^{2+} can also be accumulated by the mitochondria (66,299).

Various events accompany or result from Ca transport into mitochondria (63). The addition of Ca^{2+} to mitochondria oxidizing substrates produces a stimulation of respiration and a rapid shift in the oxidation reduction state of cytochrome b and pyridine nucleotides to a more oxidized state. The uptake of Ca^{2+} is also associated with a stoichiometric ejection of protons ($H^{+}:Ca^{2+}$; .8-1.4) and an equivalent uptake of phosphate. Whether Ca^{2+} is accumulated (1) by a carrier directly coupled to a protonated high-energy intermediate; (2) in response to a transmembrane electrochemical gradient; or (3) by some other method, is not clear. Carafoli and Sotiocasa (54) have isolated a glycoprotein which binds La and ruthenium red and possesses high affinity Ca binding sites. Whether or not this protein is involved in Ca^{2+} movement is not clear. Recent studies support the hypothesis that Ca^{2+} movement into the mitochondria depends on the electrochemical gradient of protons across the inner mitochondrial membrane (12,206,255). Ca^{2+} would be driven into the mitochondria by the mitochondrial membrane potential. This type of mechanism does not require a carrier, but Ca^{2+} may move through selective channels.

(iii) Ca^{2+} Currents

Ca^{2+} currents have been characterized by several general properties. (a) Ca^{2+} currents are inward directed; they have large positive reversal potentials (3). (b) The action potential overshoot of Ca^{2+} mediated action potentials increases with elevations in Ca_o^{2+} levels. Action potential peak amplitude changes by 29 mV per decade change in Ca_o^{2+} (3,125,242). (c) Ca^{2+} dependent currents are not eliminated by application of TTX or removal of Na_o^+ or Cl_o^-, but are blocked by La^{3+}, Co^{2+}, Mn^{2+}, Ni^{2+}, D600, verapamil and often Mg^{2+} (125,242). (d) Ba^{2+} and Sr^{2+} often substitute for Ca^{2+} as an inward current carrier (125,242). (e) Ca^{2+} channels are usually activated at potentials more depolarized than the threshold for activation of Na^+ channels and the rate of rise of Ca^{2+} dependent action potentials are usually slower than for Na^+ dependent ones (125,242).

Ca^{2+} selective channels are more primitive and possibly more ubiquitous than Na^+ selective channels (242). Hagiwara (125) has proposed that Na^+ spikes occur when conduction of a message is important and Ca^{2+} spikes when excitation is coupled to some effector mechanism. Intracellular Ca^{2+} participates in a variety of cellular functions (see Table 1).

Ca^{2+}, which enters the cell through conductance channels similar to those in other excitable tissues, is involved in intestinal slow wave generation (235,239). In this section I will briefly survey Ca^{2+} currents in excitable tissues.

A. Ca^{2+} Currents in Nerve

Baker et al. (15) and Meeves and Vogel (203) found Ca^{2+} to enter squid axons via 2 pathways during depolarization. The early rapid current had kinetics similar to the Na^+ current and was TTX sensitive. They suggested

that Ca^{2+} may have access to the Na^+ channel with one one-hundredth the affinity of Na^+ for the channel. The second component of the Ca^{2+} current had slower kinetics and a more depolarized threshold for channel activation than the first component. This current was TTX insensitive but reduced or blocked by Co^{2+}, Mn^{2+} and D600, as well as high levels of Mg_o^{2+}.

The membrane at the presynaptic terminal of the squid stellate ganglion shows an increase in permeability to Ca^{2+} ions upon arrival of presynaptic impulses (3,170). The Ca^{2+} permeability change is sensitive to Mg^{2+}, Co^{2+} or Mn^{2+} but TTX resistant. Either Sr^{2+} or Ba^{2+} can substitute for Ca^{2+} as an inward current carrier. Ca^{2+} also serves as a charge carrier in other nervous tissues: presynaptic terminal of frog neuromuscular junction (3); spinal ganglion cells of frog (163,215); secretory neuron soma of the x-organ of crayfish (148); somata of giant neurons in various molluscs; Helix (124,197), Archidoris (2,284), Aplysia (159), Anisodorous (82).

B. Ca^{2+} Currents in Muscle

In 1953 Fatt and Katz (115) found evidence for a role of Ca^{2+} ions in spike generation in crustacean muscle fibers. In normal saline the muscle fibers did not produce all or none action potentials, but following TEA application all or none action potentials were produced. They concluded that TEA reduced the outward current through the K^+ channel so a small inward Ca^{2+} current could give rise to an action potential. As in nervous tissues, Ca^{2+} currents in muscle are sensitive to changes in Ca_o^{2+}, substituted for by Ba^{2+} or Sr^{2+} and reduced or eliminated by various Ca^{2+} specific channel blockers (125,242). Aequorin and ^{45}Ca influx studies also implicate Ca^{2+} as a current carrier in muscle (140). A Ca dependent current has been found in other muscles: lobster (302) _Balanus_ _nublius_ (187);

Halocynthra (207); amphioxus (126); cardiac muscle (9,21); smooth muscle (see Section I-i).

C. Ca^{2+} Currents in Other Systems

Chemical or mechanical stimuli applied to the anterior end of the protozoan Paramecium activates Ca^{2+} selective conductance channels (105). The Ca^{2+} dependent current activates an ATP dependent process which results in reversal of the direction of ciliary beating and increases in the frequency of beating (105). This current is sensitive to removal of Ca_o^{2+} or application of Ca channel blockers. Ba^{2+} can substitute for Ca^{2+} as an inward current carrier but does not activate posterior end K^+ conductance channels which are normally activated by Ca^{2+}.

In the luminal membrane of skate electroreceptor cells a voltage dependent Ca^{2+} current has been described (74). The depolarization of a cell from the Ca^{2+} dependent current results in synaptic transmission at the basal membrane of the cell. An increase in Ca_i^{2+} is associated with activation of an outward current which is responsible for repolarization of the membrane.

Summary

The reactions which control the frequency of cat small intestinal slow waves have not yet been elucidated. Changes in extracellular Ca^{2+} levels alter slow wave frequency; hence, Ca^{2+} may be involved in the mechanism setting frequency. The purpose of this study is to determine the effects of alterations in internal Ca^{2+} levels on the frequency of slow waves. This analysis may give insight into the role of Ca^{2+} in the pacemaker process of cat small intestinal slow waves.

II. MATERIALS AND METHODS

A. Electrical and Mechanical Measurements

(1) Dissection

Intestinal muscle was obtained from the domestic cat, *Felis domestica*. Animals were kept for at least two weeks under the supervision of Mr. O. Mackey and Mrs. S. Trotter before use. Cats were anesthetized with α-chloralose (1 gm in 10 ml propylene glycol; injection: i.p.). Twenty to thirty minutes after injection, the small intestine was removed from the animal and placed into cold (3-7°C) Krebs solution aerated with 95% O_2-5% CO_2. The segments not used immediately were stored in Krebs solution at 4°C. Prior to fine dissection, a segment (6 cm long) was equilibrated in aerated warm (37°C) Krebs solution for thirty minutes.

To obtain small pieces of musculature the following procedure was used. A glass rod is inserted into the intestinal lumen. The rod diameter should be such that the segments fit loosely over the rod (Figure 1). The rod with intestine is suspended in a large Krebs-filled dissection dish. The mesentary is then removed from the muscle with fine dissection scissors. Care must be taken to assure that the mesentary is removed with little damage to the muscle layers. The segment is then gently removed from the rod and a 23 cm long, curved surgical hemostat is clamped to one end of the muscle and the segment is folded back on itself, everting the segment of gut. The everted segment is removed from the hemostat and gently placed back on the glass rod and the preparation is reimmersed in the Krebs-filled dissection dish. A longitudinal cut is made through the mucosa with a clean sharp razor blade. All attempts should be made to ensure that the razor blade makes a surface cut and does not penetrate the circular muscle. Edges of the mucosa are then pulled back with fine dissection forceps and the mucosa gently separated from the segment; connective tissue strands

Figure 1. Segment of cat small intestine mounted on a dissection rod. A region of longitudinal muscle is peeled back exposing the underlying circular muscle layer. (Photo courtesy of B. Nelson)

from the muscle to the mucosa are cut. Once the mucosa is removed the segment of muscle is taken from the rod and everted by the same procedure as outlined above. The segment is cut to 4 cm long and mounted on the tube electrode (part 3).

The tube electrode is mounted in the recording chamber for microelectrode impalements of the longitudinal muscle. For circular muscle impalements, two types of changes are implemented: (i) a small window is made in the longitudinal muscle and the microelectrode is inserted; (ii) the mucosa free muscle is left everted and placed over the tube electrode. A slit is made in the muscularis mucosa exposing the circular muscle layer.

Recordings were also made from isolated longitudinal muscle. Isolated longitudinal muscle segments were prepared by the following procedure. Mucosa-free segments were mounted on the tube electrode, in the everted configuration. Following the removal of the outermost muscle layer,(muscularis mucosa) rings of circular muscle were removed until cylinders of isolated longitudinal muscle were present. Isolation of the longitudinal muscle, from the circular, was confirmed by dissection microscope examination. In addition, histological inspection of random muscle sections (by Mr. D. Nelson, technique) also confirmed the presence of isolated longitudinal muscle. Flat segments of isolated or intact muscle were prepared by cutting along the mesenteric border of mucosa-free segments. The flat segments were trimmed to 2 cm^2 squares.

During recording, aerated solution flowed through the recording chamber. Temperature was maintained at 35-37°C as monitored by a Yellow Springs Instruments thermometer (Model #42SC).

(2) Solutions

The composition of the various solutions used in this study are given in Table 1. Normal Krebs solution was aerated with 95% O_2-5% CO_2. Krebs solution buffered by MOPS buffer was aerated with 100% O_2 or air. All solutions were maintained at pH 7.4. Experimental solutions contained the agent to be examined in addition to the normal ionic composition. Solutions used for microelectrode studies contained 150 mM sucrose which was added to the Krebs solution to prevent movement.

Drug	Source
1) D600	Knoll Pharmaceutical, West Germany
2) verapamil	Knoll Pharmaceutical, New Jersey
3) EGTA	Sigma Chemical Company, St. Louis, Mo.
4) dibutyrl cAMP	Sigma Chemical Company, St. Louis, Mo.
5) methylene blue	Eastman Organic Chemicals, Rochester, New York
6) ouabain	Sigma Chemical Company, St. Louis, Mo.
7) MOPS	Sigma Chemical Company, St. Louis, Mo.
8) eserine	Sigma Chemical Company, St. Louis, Mo.
9) chlorotetracycline	Sigma Chemical Company, St. Louis, Mo.

(3) Equipment and Recording

(a) Mechanical

Contractions were recorded by an isometric tension transducer (Grass Inst. Model #FT03C). A thin piece of surgical thread was attached to the transducer and a hook made from an insect dissection pin (Essexs Ltd.) was attached to the other end of the thread. The hook was inserted into the muscle and the transducer positioned until there was light stress on the

Table 1. Composition of solutions used (in mM).

	Na	K	Ca	Mg	Cl	HCO_3	H_2PO_4	Glu	MOPS	EGTA
Krebs	142.3	5.9	2.5	1.2	130.6	23.8	1.2	5.5	--	--
MOPS Krebs	140.3	5.9	2.5	1.2	153.6	--	--	5.5	4.0	--
Ca^{2+} Free Krebs	142.3	5.9	--	1.2	130.6	23.8	1.2	5.5	--	5-10
0 Ca^{2+} Krebs	142.3	5.9	--	1.2	130.6	23.8	1.2	5.5	--	--

thread. Contractile activity was displayed by a Grass ink writing polygraph (Model #79D).

(b) Extracellular Electrical

Electrical events were recorded extracellularly by tube electrodes (Figure 2), pressure electrodes (Figure 3) and suction electrodes (Figure 4). Each electrode type is described in depth in its corresponding figure legend. Signals from the electrodes were fed to a Grass ink writing polygraph (Model 79D). The output of the polygraph driver preamplifier was fed to an oscilloscope (Hewlett Packard Model #135A). The output of the oscilloscope was fed through a FM recording adapter (A.R. Vetter Co. Model 2D) and after processing recorded on magnetic tape (Sony Model TC353-D).

(c) Intracellular Recording

The following procedure was followed for microelectrode fabrication and recording. Omega dot (Frederick haer and Co.; #30-31-1) microelectrode glass, cleaned with aqua regia solution (1 HNO_3:4 HCl) then flushed with water and evaporated with ethanol, is cut in half with a fine cutting stone. Two microelectrodes are pulled from each half piece of capillary on a David Kopf vertical microelectrode puller (#700C). The electrodes are then scored three-quarters of the way down the shank and broken at the score mark; the excess capillary is discarded. The electrode is filled with 3 M KCl and a thin tungsten wire (Philips Elmet Corp.: 1.88 mls) inserted into the electrode lumen. The lumen is then sealed with bees wax and the electrode is suspended by 7 cm of wire, electrically insulated from and mounted to an Aus Zähne micromanipulator (Figure 5) (305). The tungsten wire is connected to the input of a high impedance, capacity compensated preamplifier.

Figure 2. Description of the tube electrode. The tube electrode consists of a plexiglass rod with three longitudinally directed holes drilled into one of its ends. The holes are continuous with radial holes drilled from the rods surface. The radial directed holes lay in a line on the rods surface and constitute the recording sites. The recording loci were 7 and 8 mm apart. Each hole is filled with saline. Ag - AgCl wires, jacketed over a portion of their length with polyethylene tubing, are inserted into the holes at the blunt end of the rod. A tight fit between the polyethylene tubing and the holes give adequate electrical insulation. The other end of the tube electrode is tapered to a smooth tip over which the segments of muscle can be slid. The silver wires are connected to the preamplifier on a Grass polygraph which displays the signals. The bath is grounded by a coiled chlorided silver wire.

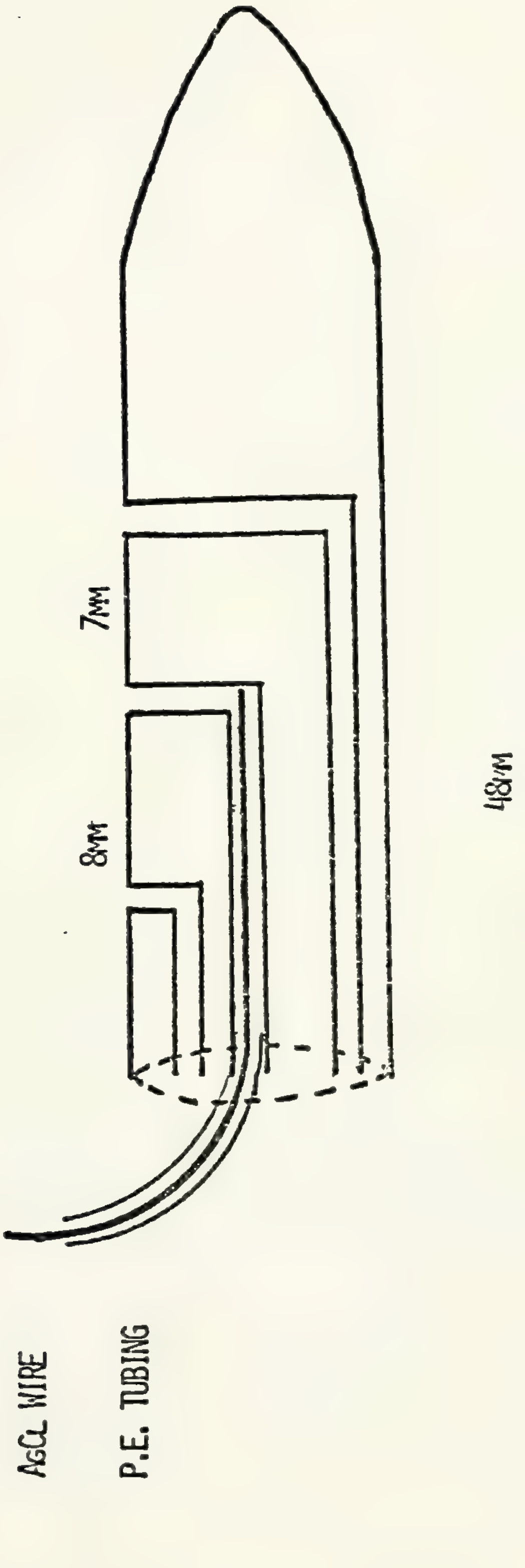

AgCl WIRE
P.E. TUBING
8mm
7mm
48mm

Figure 3. Description of the pressure electrode. The electrodes are drawn from 1-3 mm O.D. glass tubing (Kimex) very slowly over a small flame. The ends of the electrodes are fire polished to a smooth surface. Tip diameters are approximately 500 microns, ranging from 300 to 700 microns. The electrodes are filled with 0 Ca^{2+} saline containing 2 g Agar per 100 mls saline. The Agar-saline mixture is brought to a boil and then the electrodes are filled by suction. Suction is applied through a syringe which has a short segment of polyethylene tubing connecting the capillary tube to the needle. The tip of the pressure electrode is immersed in approximately 30 mls of the Agar-saline solution when suction is applied. A segment of chlorided silver wire is then inserted into the electrode.

The electrode can now be connected to an aligator clip approximately 7 cm from the electrode tip. The aligator clip is mounted on a plexiglass rod which is attached to a micromanipulator. The electrode is then lowered onto the preparation, applying light pressure on the preparation. The chlorided silver wire is connected to the input of the Grass polygraph.

The bath is grounded by a coiled chlorided silver wire.

The polarity and configuration of potentials recorded with pressure electrodes are similar to those recorded intracellularly.

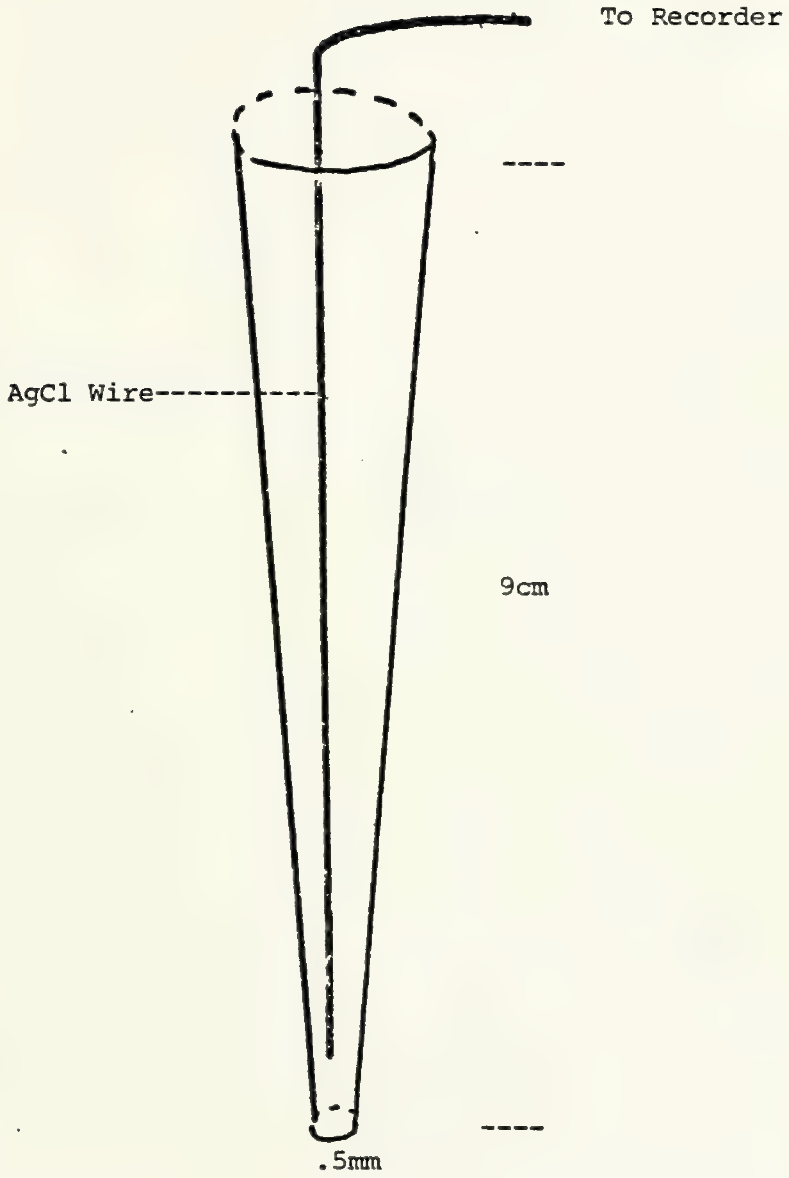

To Recorder
AgCl Wire
9cm
.5mm

Figure 4. Description of the suction electrode. In constructing a suction electrode a drawn capillary tube, as described in the legend for Figure 3, is inserted over a metal syringe needle. A chlorided silver wire is threaded through the needle to within 5 mm of the capillary tip. The chlorided wire is bent such that it touches the surface of the needle. The needle, wire, and capillary assembly is attached to a 5 ml syringe; this assembly constitutes the suction electrode. The suction electrode is mounted to a micromanipulator and lowered into the organ bath. Suction is applied from the syringe and the electrode is filled with 1 ml of saline taking care that there are no bubbles in the capillary tube. The electrode is gently lowered onto the surface of the preparation and suction is applied through the syringe. An alligator clip is attached to the metal needle and connected thru a banana lead to the input of a Grass polygraph. The bath is grounded by a coiled chlorided silver wire.

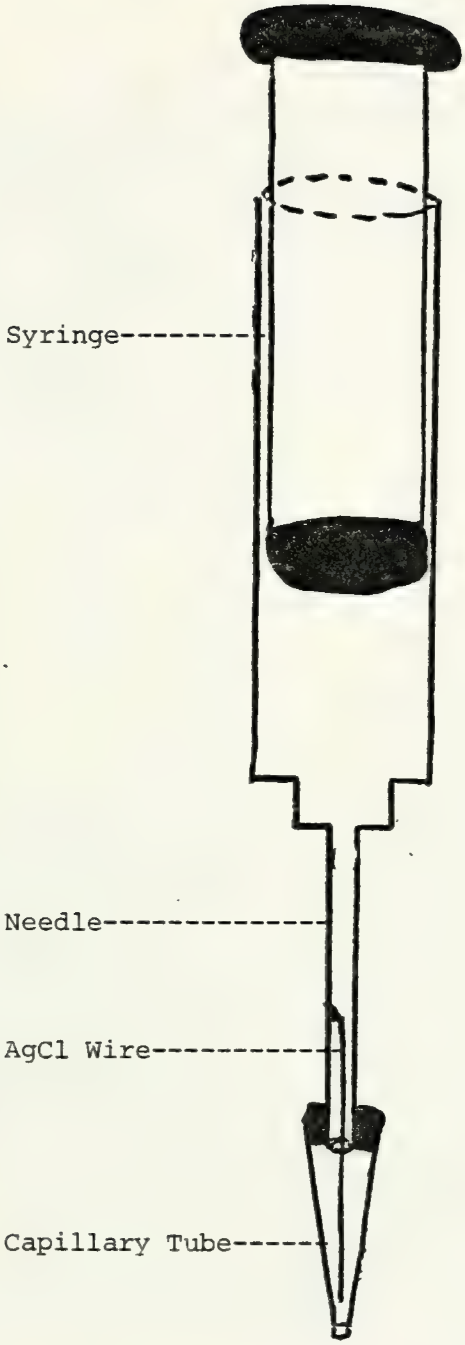

Syringe
Needle
AgCl Wire
Capillary Tube

Figure 5. Picture showing a segment of cat small intestine mounted in the recording chamber on a tube electrode. A "floating" microelectrode is impaling the segment. (Photo courtesy of B. Nelson)

The preamplifier output is fed to the second channel of the oscilloscope and the output of the oscilloscope fed to a Grass polygraph and Sony tape recorder via the FM adapter.

The electrode is now lowered into the recording chamber. Electrode tip resistances are measured (functional electrodes range from 40-100 MΩ). The electrodes are then lowered until they are just above the surface of the preparation. The base plate is tapped gently causing the electrode to "pop" into the cell. Changes in voltage were also monitored by a voltage controlled oscillator.

(d) Chamber

The recording chamber was a cubical well (4.6 cm x 2.7 cm x 1.9 cm) machined from a plexiglass block. The chamber sat on a plexiglass rectangular base maintained at a constant temperature by a Haake temperature bath (Model: FS). The base was mounted to the micromanipulator.

(e) Experimental Considerations

In determining the success of microelectrode impalements 3 criteria were adhered to. First there had to be a rapid change in membrane potential. Secondly, the membrane potential had to be stable for at least 25 seconds, and finally the membrane potential had to be greater than 40 mV. This last criteria was to insure that the electrode was in a viable cell.

B. Optical Studies

(1) Solutions and Dissections

Solutions and dissection procedures were as in part A.

(2) Chamber

The chamber was a plexiglass cubical 10 cm x 5 cm x 2 cm. Five cm from either end was a plexiglass mount into which the tube electrode was inserted. The chamber was attached to a mobile verner on the base of a microscope. Heated, aerated solutions flow continuously through the chamber by means of a peristaltic pump (Markman). Solutions in the recording chamber were at approximately 34°C (Yellow Springs Instr. Model #42SC).

(3) Electrical Equipment

Electrical recordings were with tube and suction electrodes (see section A3). Signals from the electrodes were fed to a Grass ink writing polygraph. The polygraph output was fed to an oscilloscope (Tektronix 502A) and the oscilloscope output to one channel of a computer of average transients (CAT) (Technical Measurements Corp. Model #400C) (Figure 6).

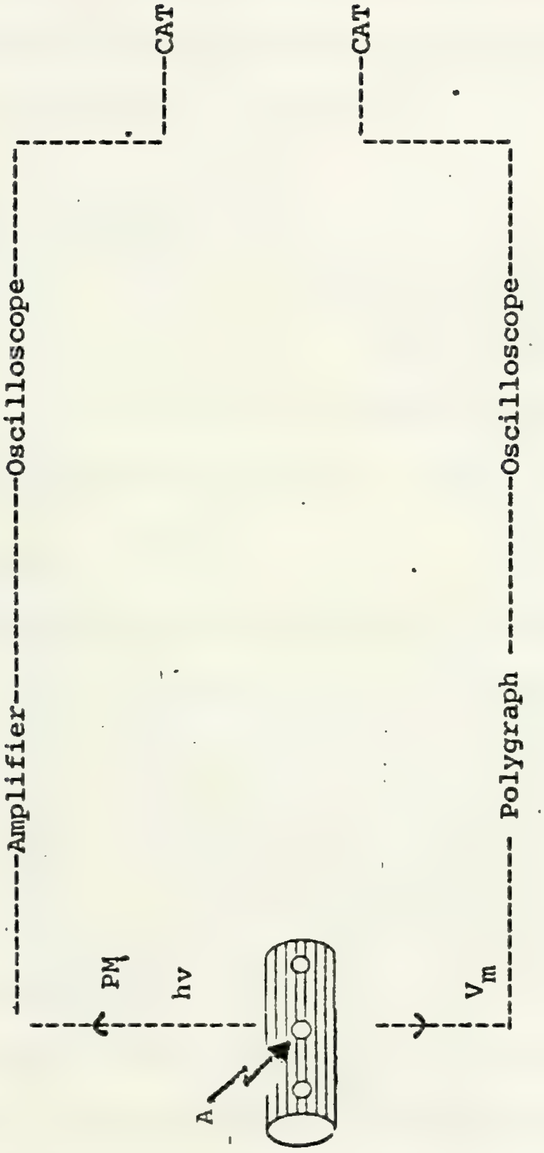
(4) Optical Equipment

A small spot (∿1-2 mm) of near ultraviolet light ($340 < \lambda < 380$ nm) from a xenon arc source (Ushio Electric Inc. DSB 151A) was beamed onto the surface of the preparation from a fiber optic guide. The spot illuminated the area of tissue corresponding with the region from which the voltage signal was being recorded (Figure 6). The fiber optic guide made an angle of approximately 45° with the preparation.

Light emissions were picked up by a light guide mounted to a microscope objective. The light passed through a 400 nm cut off filter and either a 530 nm (for chlorotetracycline studies) or a 460 nm (for NADH studies) interference filter before reaching the photomultiplier tube.

An RCA 931 photomultiplier tube was mounted in the shaft of a microscope (the chamber was mounted to the microscope platform - see B2) which

Figure 6. Apparatus for measuring fluorescence and electrical activity simultaneously. (A) is the excitation beam which makes an angle of approximately 45° with the preparation. The photomultiplier tube (PM) picks up light signals (hr) in the same region where voltage (V_m) is extracellularly recorded. The output of the PM is fed through an amplifier to an oscilloscope and from there to one channel of a computer of average transients (CAT). The voltage signal is recorded on the polygraph and taken from the output of the polygraph to the second channel of the oscilloscope and from there fed to the second channel of the CAT.


A
PM
hν
V_m
Amplifier
Oscilloscope
CAT
Polygraph
Oscilloscope
CAT

was perpendicular to the preparation. The photomultiplier tube photocathode was maintained at -800 V by a stabilized high voltage power supply (Hewlett Packard Model 6515A). The output from the anode was coupled through a low pass filter to the second input of the oscilloscope. The output of the oscilloscope was fed to the second channel of the polygraph and computer of average transients (CAT) (Figure 6).

(5) Data Collection and Analysis

The computer of average transients was manually triggered during a constant phase of the slow wave. Between 10-60 slow waves were averaged for each record. Photographs were taken from the CAT display with a 35 mm (Cannon AE-1) and an oscilloscope (H.P. 196B) camera.

C. Oxygen Studies

For hypoxia experiments solutions were aerated with a N_2-CO_2 (95%-5%) gas mixture. Oxygen concentrations were recorded with an oxygen monitor (Yellow Springs Instruments - Model 55) and then converted directly to parts per million (ppm) oxygen with an oxygen meter (Yellow Springs Instruments - Model 54). The recording apparatus is illustrated in Figure 7.

D. Electron Microscopy

The electron micrographs used in this study were generously provided by Dr. A. Taylor. The procedure for preparation of the micrographs was the same as described by Taylor *et al*. (282).

E. Miscellaneous Preparations

Segments of cat vena cava, esophagus, stomach, colon, bladder, and uterus were removed and prepared by the same procedure as for cat small intestinal

Figure 7. Oxygen recording set-up.

A - Stock Solution

B - Peristaltic Pump

C - Muscle Chamber

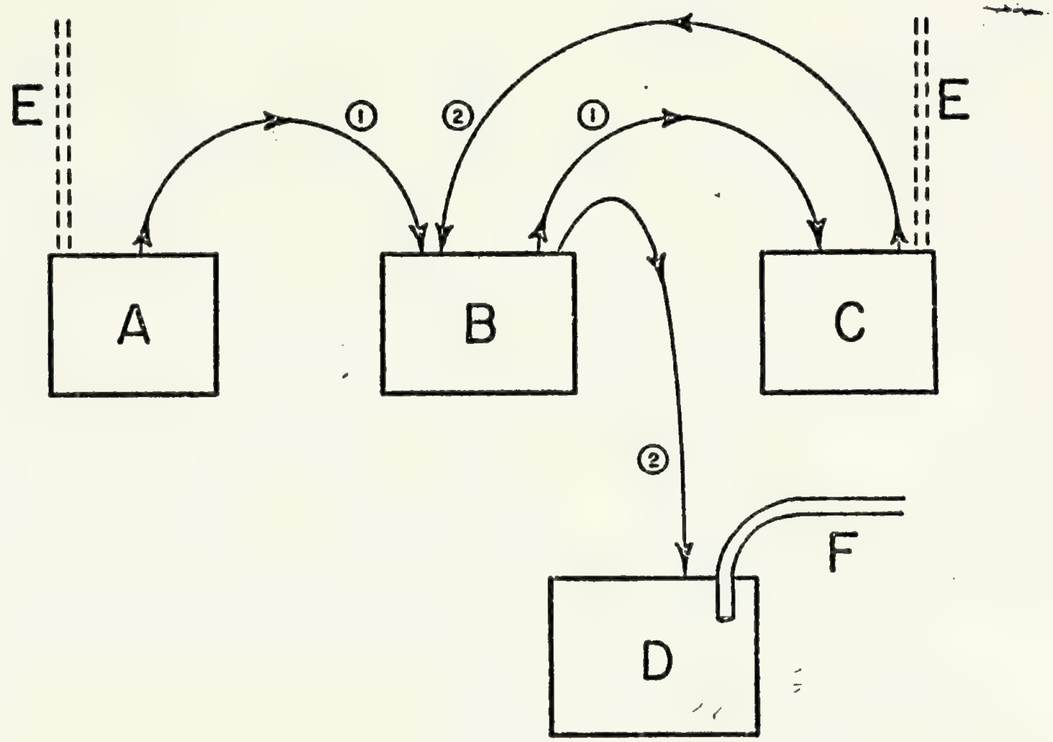
D - Oxygen Monitor

E - Gas Lines

F - Vacuum Hose

Arrows denote direction of flow of solutions. The same encircled number represents a continuation of the same piece of tubing. Aerated solutions are brought from the stock bottle to the muscle chamber via a peristaltic pump. Solution is taken from the muscle chamber to the oxygen monitor. The oxygen monitor is calibrated with an oxygen meter.

Oxygen Recording Set-up



segments. Rats were anesthetized by diabutol or killed by concussion and the small intestine was removed by an identical procedure as for other preparations.

III. RESULTS AND DISCUSSION

A. The Effects of Alterations in Internal Ca^{2+} Levels in Slow Waves

Summary

(1) Reductions in Ca_i^{2+} produced a decrease in slow wave frequency characterized by a prolongation of the slow wave diastolic phase. Identical results were observed following: reductions in Ca_o^{2+}, addition of Ca channel blockers, increases in Mg_o^{2+} or increases in internal pH, all treatments which decrease Ca_i^{2+}.

(2) Elevations in Ca_i^{2+}, via an increase in Ca_o^{2+}, initially produced an increase in frequency, while further elevations produced a decrease. The frequency changes were characterized by shortening of the diastolic phase of the slow wave.

(3) CTC fluorescence studies show an oscillation in Ca_i^{2+} occurs during the course of slow waves.

A. The Effects of Alterations in Internal Ca^{2+} Levels on Slow Waves

(i) Slow Wave Activity

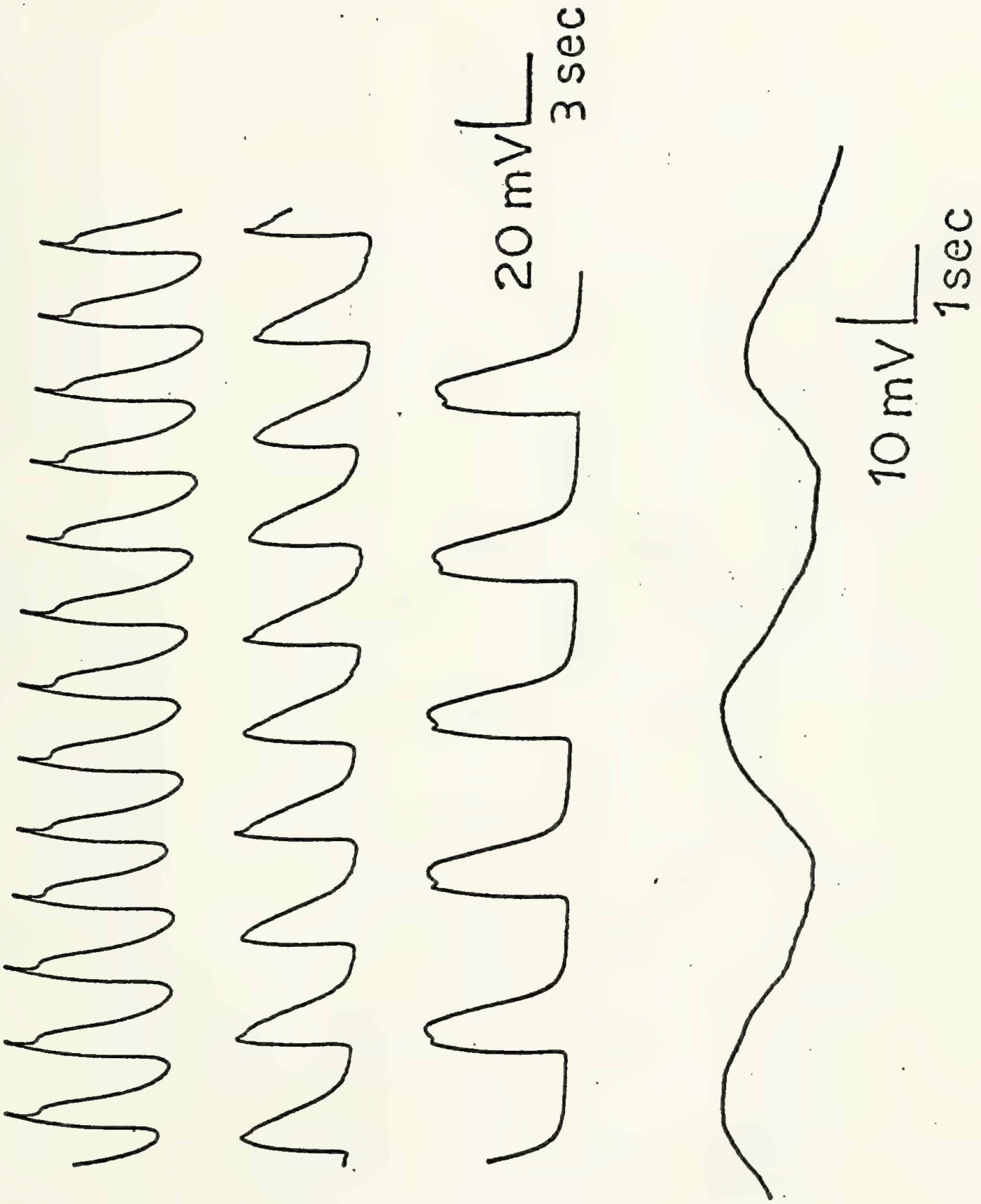
Spontaneous slow waves recorded with microelectrodes from intact and isolated longitudinal muscle are illustrated in Figure 1. Table 1 summarizes the measurements of slow wave amplitude and membrane resting potential. Slow waves recorded from the duodenum were characterized by faster frequencies and shorter diastolic phases (see Figure 1) than those recorded from the jejenum or ileum. Ileal slow waves occurred at a slower frequency and had more prolonged diastolic phases than slow waves recorded from the jejenum. As observed by Connor _et_ _al_. (80), the amplitudes of slow waves recorded from isolated longitudinal muscle were smaller than those recorded from intact muscle segments; although resting potentials were nearly identical in intact and isolated segments. This reduction in slow wave amplitude is due to removal of reinforcing (or amplifying) current spread from the circular muscle layer (80).

The values for mean slow wave amplitude and mean resting membrane potential given in Table 1 correlate well with other published values (77,79,80, 106,107).

(ii) Effects of Reduction in Ca_o^{2+}

Reduction in Ca_o^{2+} decreased the frequency and amplitude of slow waves recorded from intact cat small intestinal muscle (80). Microelectrode recordings from intact longitudinal muscle in normal saline and the activity after a 3 minute exposure to 0 Ca^{2+} saline (see Methods) (n=10) are illustrated in Figure 2. During exposure to 0 Ca^{2+} saline slow wave amplitude and frequency declined in all preparations. The reduction in slow wave frequency was characterized by a prolongation of the diastolic region

Figure 1. Intracellular recordings of slow waves in longitudinal muscle. The upper 3 records are recordings from the duodenum, jejenum and ileum (respectively) of intact cat intestine. The fourth record is from isolated longitudinal muscle of the jejenum. All recordings are from different cats.

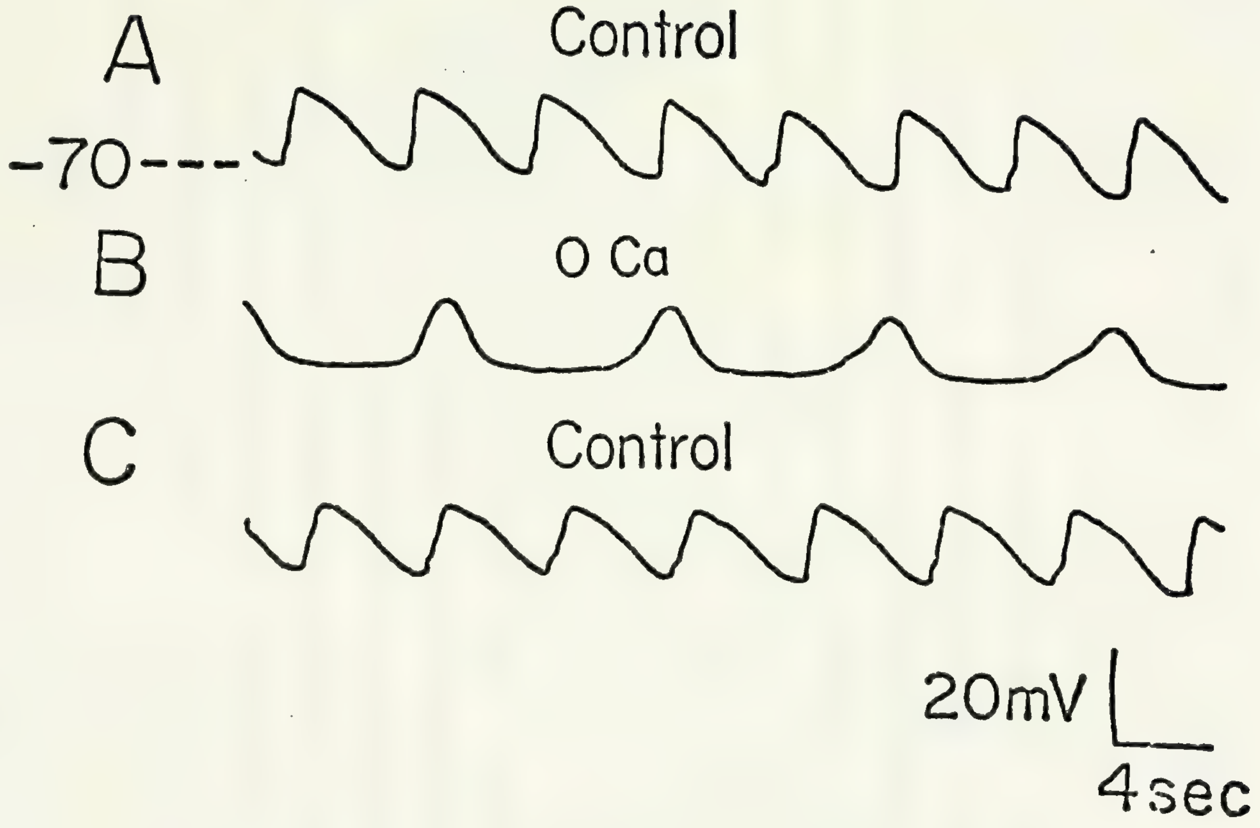

20 mV
3 sec
10 mV
1 sec

1

Table 1. Electrical Parmaeters Measured Intracellularly in the Small Intestine of the Cat.

	RESTING MEMBRANE POTENTIAL	SLOW WAVE AMPLITUDE	N
LONGITUDINAL MUSCLE			
INTACT SEGMENTS	68.9 ± 9 MV	23.7 ± 10 MV (RANGE 6-44 MV)	99
ISOLATED SEGMENTS	64.7 ± 9 MV	13.7 ± 7 MV (RANGE 2.5-25 MV)	63
CIRCULAR MUSCLE			
INTACT SEGMENTS	63.6 ± 10 MV	18.5 ± 9 MV (RANGE 3-42 MV)	74

Figure 2. Effects of 0 Ca^{2+} saline on slow waves in intact longitudinal muscle. Trace (B) represents the effects of 3 min incubation in 0 Ca^{2+} saline. In traces (B) and (C) there was no resting potential change from trace (A). All traces are from the same cell. Return to control (C) is after 4 1/2 min in normal saline. (Recorded with microelectrodes)

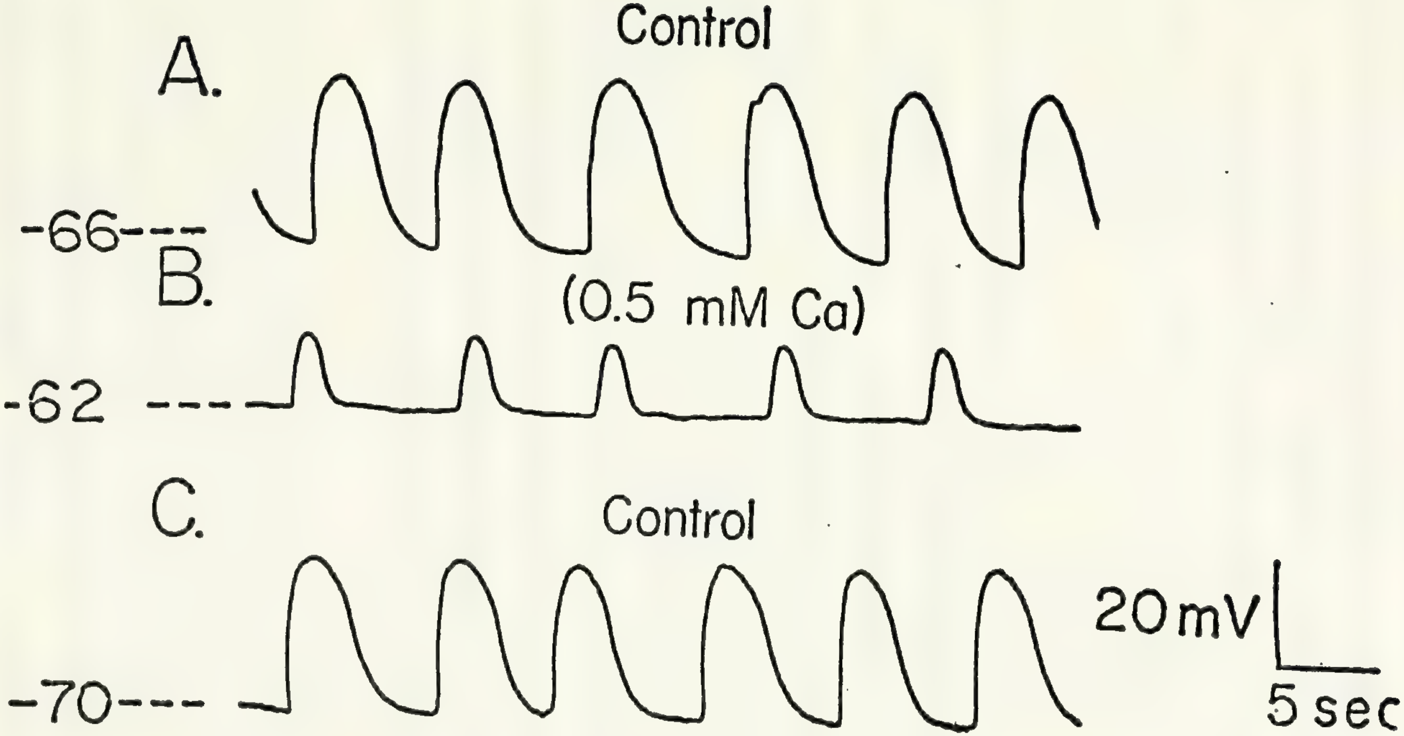

A
Control
-70
B
0 Ca
C
Control
20mV
4sec

of the slow wave, as compared with control slow wave activity. Following return to normal Ca^{2+} levels, slow wave frequency returned to control values as did the duration of the slow wave diastolic phase. In five preparations there was no change in resting membrane potential during exposure to 0 Ca^{2+} saline. In 5 other preparations, a small depolarization resulted from exposure to 0 Ca^{2+} saline but never did the depolarization exceed 6 mV.

The reduction in slow wave amplitude following decreases in extracellular Ca^{2+} levels is mediated by the circular muscle layer (80). Support for this is, that when recordings were made in isolated longitudinal muscle there was no decrease in slow wave amplitude following removal of Ca_o^{2+}. The decline in slow wave amplitude resultant from exposure to 0 Ca^{2+} saline, was dependent on the control amplitude of slow waves. When amplitudes were large (>30 mV), a larger percent reduction in slow wave amplitude accompanied decreases in Ca_o^{2+} than when slow waves were small (<20 mV). The effects of .5 mM Ca_o^{2+} (n=4) on slow waves recorded from intact longitudinal muscle are illustrated in Figure 3. The decrease in slow wave frequency was not as great as in 0 Ca^{2+} saline, although the diastolic phase of the slow wave was still markedly prolonged as compared to the control. The amplitude decline in this preparation was much more striking than in Figure 2, since the control slow waves in Figure 3 were substantially larger than those in Figure 2.

The effects of reductions in Ca_o^{2+} levels on slow wave frequency were not equivalent in the various regions of the intestine. During incubation

Figure 3. Effects of .5 mM Ca_o^{2+} on slow waves in intact longitudinal muscle. Trace (B) represents the effects of 5 min incubation in .5 mM Ca_o^{2+}. Return to control (C) is after 10 min in normal saline. Traces (A) and (B) are from the same cell, (C) is from a different cell in a nearby region. (Recorded with microelectrodes)


Control
A.
-66
B.
(0.5 mM Ca)
-62
C.
Control
-70
20 mV
5 sec

in low Ca^{2+} saline, the frequency of duodenal slow waves was reduced much more than either jejenal or ileal slow waves. Slow waves recorded from the jejenum were affected more than those recorded from the ileum.

To determine if the effects of Ca_o^{2+} reduction were due to alterations in extracellular or intracellular Ca^{2+}, several types of experiments were performed using agents expected to affect Ca_i^{2+} levels without disturbing extracellular levels.

(a) Effects of Ca Channel Blockers

Cobalt (Co^{2+}), verapamil, manganese (Mn^{2+}) and D600 block the entry of Ca^{2+} into cells (3,125,193,194,196,242) and might, therefore, be expected to lower Ca_i^{2+}. Control slow wave activity recorded from intact longitudinal muscle and the resulting activity following 3 and 5 minute exposure to 5 mM Co^{2+} (n=4) is illustrated in Figure 4. Slow wave amplitude and frequency declined in a manner similar to that observed during incubation in 0 Ca^{2+} saline, in that the decreased frequency resulted primarily from a prolonged diastolic phase. Application of 10 µM verapamil (Figure 5) (n=4), 1 mM Mn^{2+} (n=2) or 1 µM D600 (n=2) had identical effects on slow waves as treatment with Co^{2+}.

Co^{2+} and Mn^{2+} are divalent cations, consequently the effects of Co^{2+} or Mn^{2+} on slow wave activity could possibly result from displacement of bound Ca from external sites. Since verapamil and D600 do not resemble Co^{2+} or Mn^{2+}, but had identical effects on slow wave activity, it is concluded that the effects of the Ca channel blockers, on slow wave activity, do not result from displacement of bound Ca from external sites, but from the conventional action of limiting the influx of Ca^{2+} into cells. Since the effects of Ca channel blockers and exposure to 0 Ca^{2+} saline were identical, it is suggested that the effects of reduced Ca_o^{2+} on slow waves

Figure 4. Effects of 5 mM Co^{2+} on slow waves in intact longitudinal muscle. Traces (B) and (C) represent 3 and 5 min incubations in 5 mM Co^{2+}. Return to control (D) is after 11 min in normal saline. Traces (A), (B) and (C) are from the same cell, (D) is from a different cell in a nearby region. (Recorded with microelectrodes)

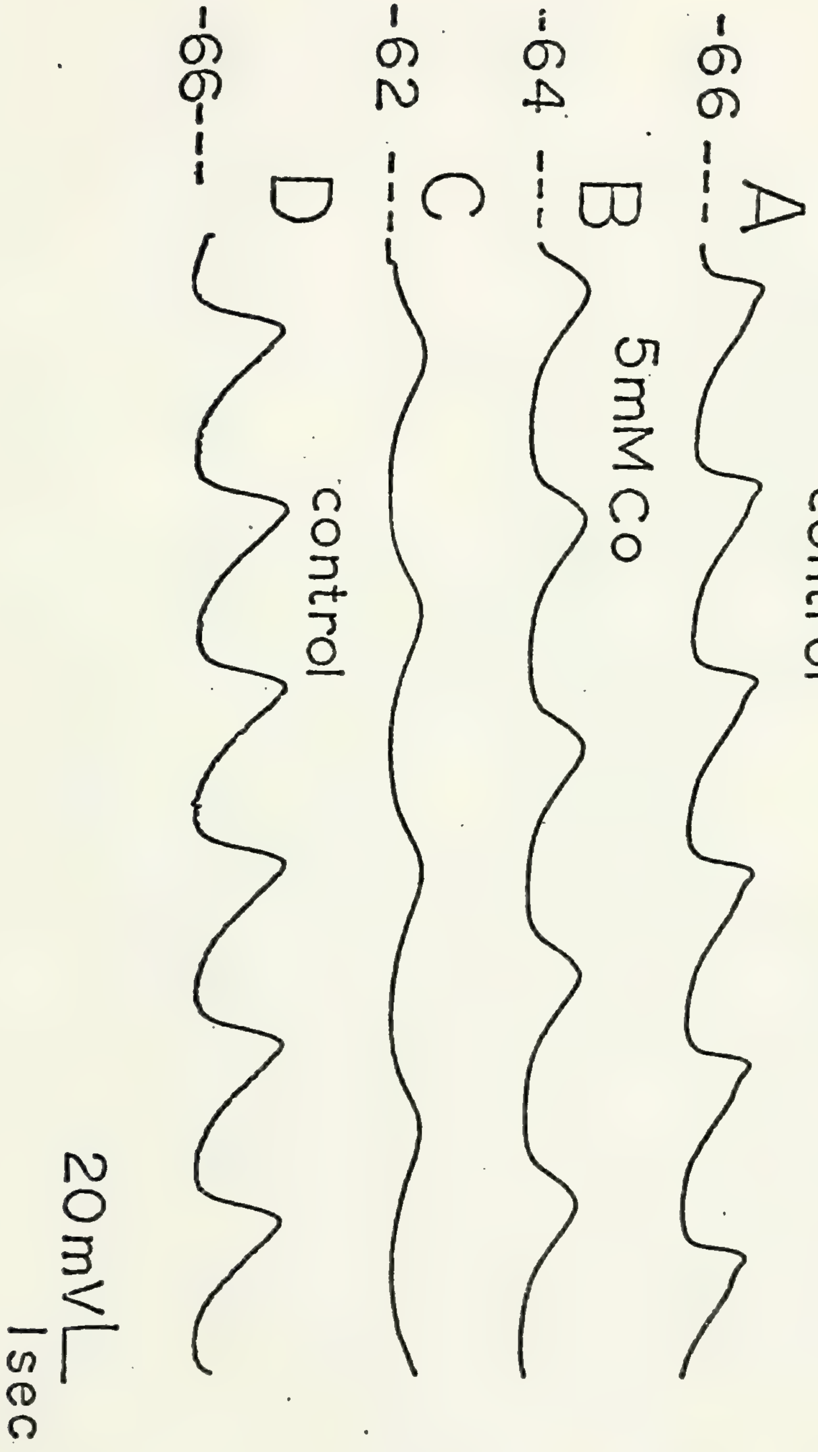
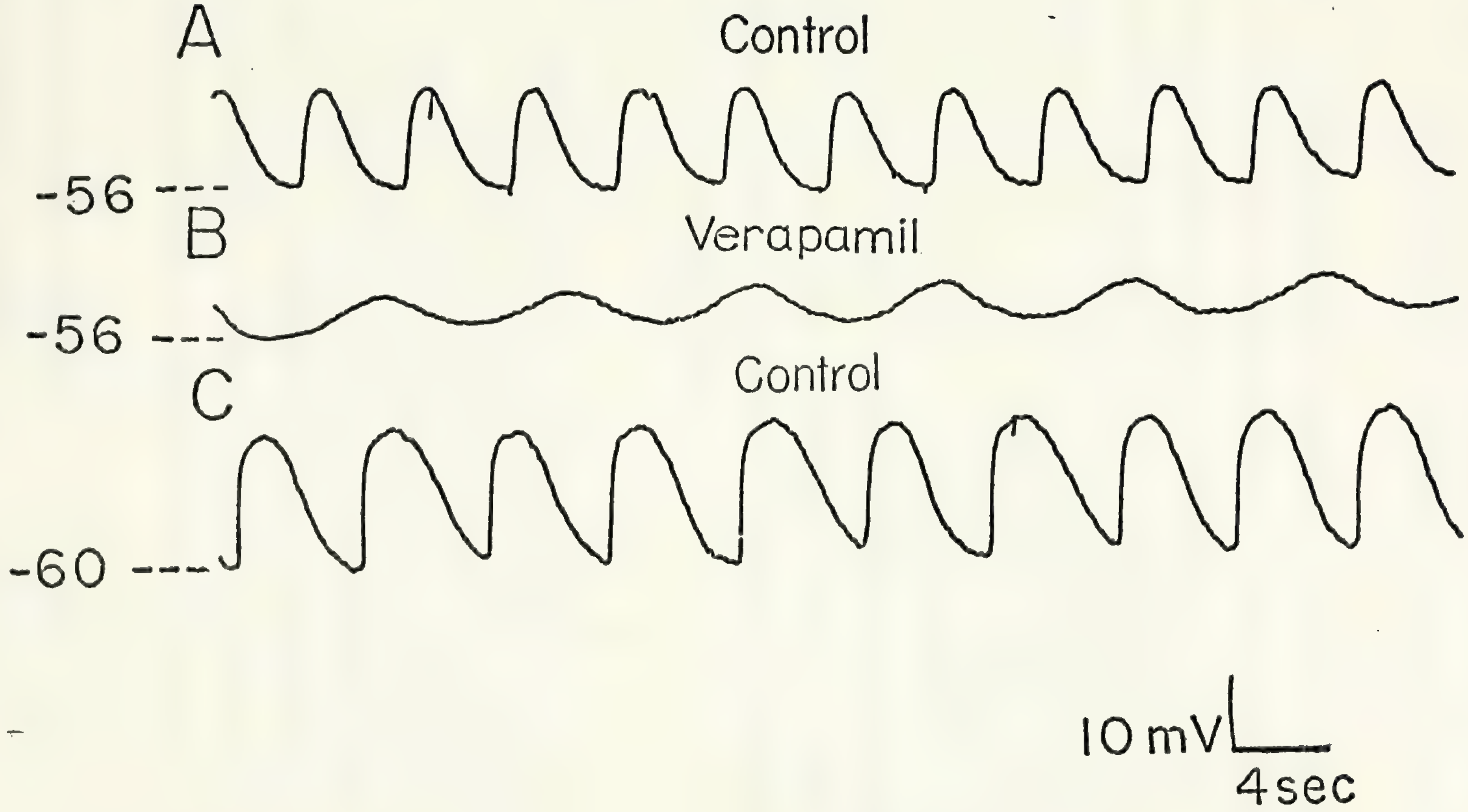

-66
A
control
-64
B
5mM Co
-62
C
-66
D
control
20mV
1sec

Figure 5. Effects of 10 µm verapamil on slow waves in intact longitudinal muscle. Trace (B) represents 5 min incubation in 10 µM verapamil. Return to control (C) is after 10 min in normal saline. Traces (A) and (B) are from the same cell, (C) is from a different cell in a nearby region. (Recorded with microelectrodes)


A
Control
-56
B
Verapamil
-56
C
Control
-60
10 mV
4 sec

result from a decrease in Ca^{2+} influx and therefore, decreased internal, not external, Ca^{2+} levels. Although an equilibrium between Ca_o^{2+} and Ca_i^{2+} exists.

Since circular muscle activity is Ca dependent (75,80,178), blocking the influx of Ca^{2+} may affect slow wave frequency by reduction of circular muscle input to the longitudinal muscle slow wave generating mechanism. To eliminate this possibility, segments of isolated longitudinal muscle were tested. Addition of 10 μM verapamil to the bathing solution slightly decreased slow wave frequency by causing a prolongation of the diastolic phase (n=3) (see Figure 6). Connor et al. (80) showed reduction in Ca_o^{2+} has equivalent effects on the frequency of slow waves recorded from either isolated or intact longitudinal muscle. From these results, it is concluded that decreasing Ca_o^{2+} exerts its effects on slow wave frequency via the longitudinal, not circular, muscle layer.

(b) Magnesium

Mg^{2+} competes with Ca^{2+} for access to membrane channels and therefore, limits the influx of Ca^{2+} into cells (125,242). The effects of increasing Mg_o^{2+} to 20 times normal levels (n=3) are illustrated in Figure 7. Slow wave frequency was decreased, with the slow waves characterized by prolonged diastolic phases. Slow wave amplitude was also reduced during exposure to high levels of Mg_o^{2+}. The effects of 20 times normal Mg_o^{2+} levels on slow wave activity suggest a competition of Ca^{2+} with Mg^{2+} for entry into cells.

In solutions containing diminished Ca^{2+} levels, smaller increases in Mg_o^{2+} were sufficient to produce comparable effects on slow waves. In solutions containing .5 mM Ca_o^{2+}, increasing Mg_o^{2+} to 10 times normal levels (n=6) resulted in a large decrease in slow wave amplitude and frequency (Figure 8).

Figure 6. Effects of 10 μM verapamil on slow waves in isolated longitudinal muscle. Trace (B) represents 4 1/2 min incubation in 10 μM verapamil. Traces (A) and (B) are from the same cell. (Recorded with microelectrodes).

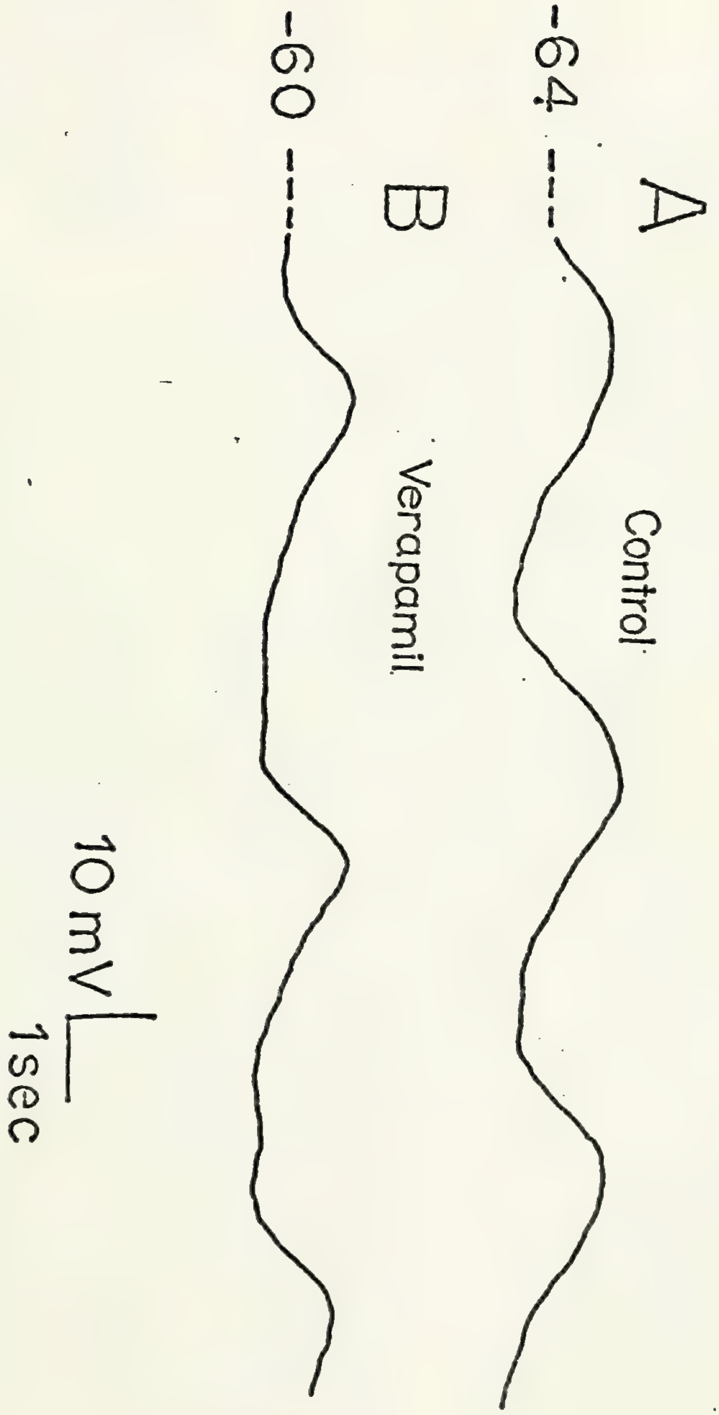

A
-64
Control
B
-60
Verapamil
10 mV
1sec

Figure 7. Effects of 20 times normal Mg_o^{2+} on slow waves in intact longitudinal muscle. Trace (B) represents 3 min incubation in 20 times normal Mg_o^{2+} levels. Return to control (C) is after 7 min in normal saline. There was no resting potential change in traces (B) or (C) from trace (A). All traces are from the same cell. (Recorded with microelectrodes)

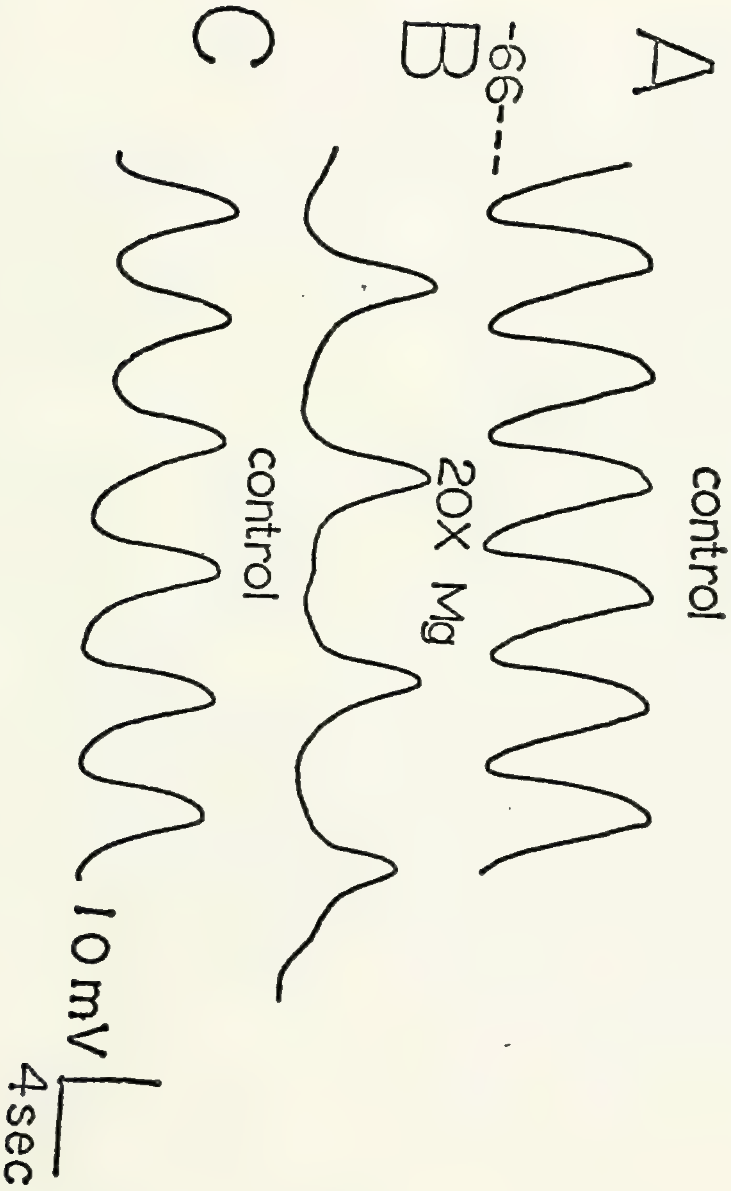
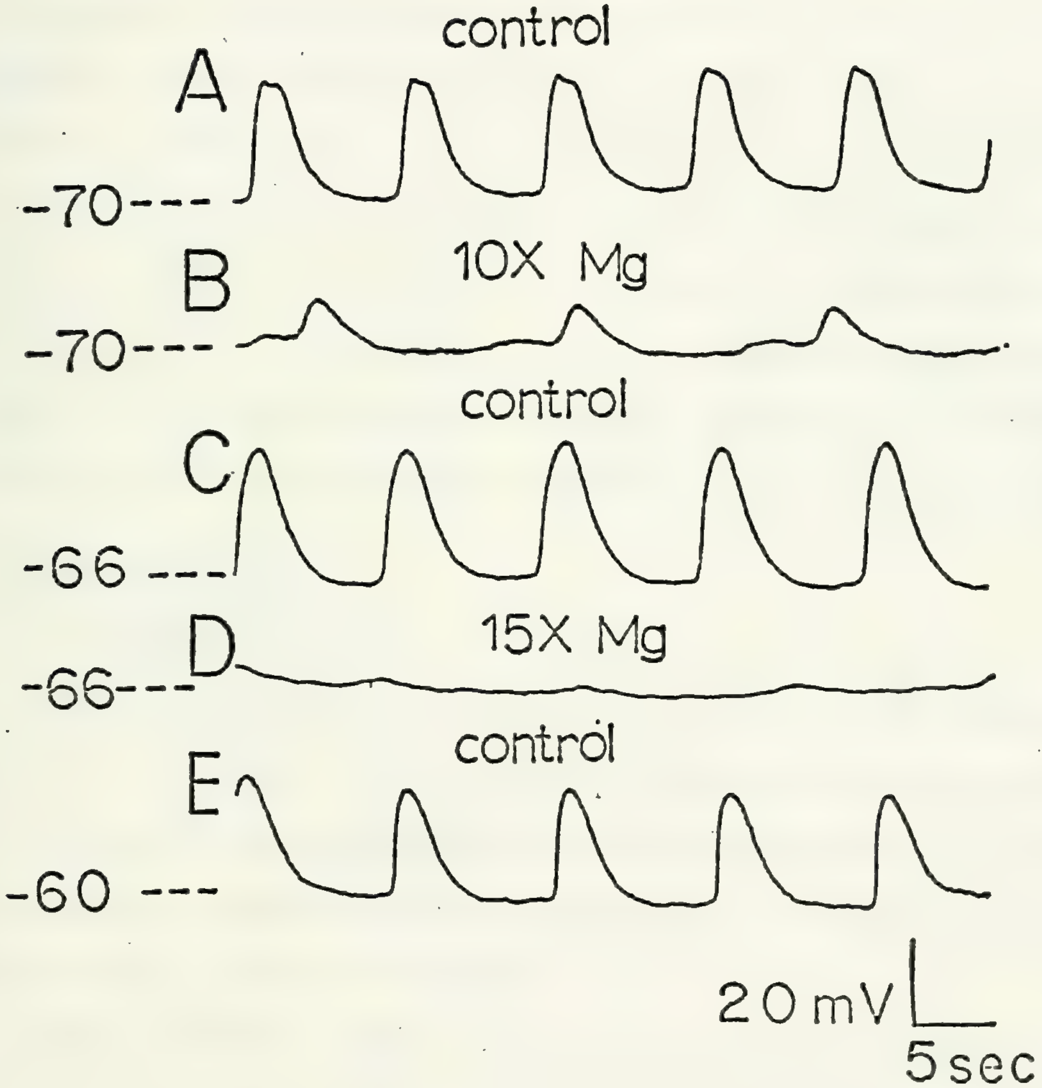

A
control
-66---
B
20X Mg
C
control
10 mV
4 sec

Figure 8. Effects of 10 and 15 times normal Mg_o^{2+} levels on slow waves in intact longitudinal muscle. All solutions, control and experimental, contained .5 mM Ca_o^{2+}. Trace (B) represents 4 min incubation in 10 times normal Mg_o^{2+}. Trace (D) is after 3 min incubation in 15 times normal Mg_o^{2+}. Traces (A) and (B) are from the same cell; traces (C) and (D) are from the same cell. Trace (E) is recorded from a different cell than either traces (A) and (B) or (C) and (D). All recordings are from the same region of the same preparation. (Recorded with microelectrodes)


control
A
-70---
B
10X Mg
-70---
control
C
-66 ---
D
15X Mg
-66---
control
E
-60 ---
20 mV
5 sec

Increasing Mg_o^{2+} to 15 times normal levels reversibly eliminated slow waves. It is significant that when slow waves were eliminated by high levels of Mg_o^{2+}, the membrane potential remained at the diastolic level.

(c) pH

Changes in internal pH have been correlated with changes in Ca_i^{2+} levels. Increasing internal pH is associated with a decrease in Ca_i^{2+} (2,28) while a decrease in internal pH is associated with an increase in Ca_i^{2+} (2,173,199). Addition of 10 mM NH_4Cl to the bathing medium (n=4), a treatment which may increase internal pH (decrease Ca_i^{2+}), resulted in a decrease of slow wave frequency with slow waves characterized by a prolonged diastolic phase (Figure 9). Prolonged exposure to the NH_4Cl solution (i.e., >10 min) irreversibly eliminated slow waves. Decreases in pH are discussed later.

(iii) Increases in Ca_i^{2+}

(a) Increases in Ca_o^{2+}

Increasing Ca_o^{2+}, up to 4 times normal levels (n=4), resulted in increases in slow wave frequency. As illustrated in Figure 10, the frequency increase was characterized by a shortening of the diastolic phase of the slow wave with respect to control activity. Accompanying the increase in frequency was a decline in slow wave amplitude.

Further increases in Ca_o^{2+} (n=6) produced a decrease in slow wave frequency (Figure 11). In this case, the diastolic phase of the slow wave was also shortened and the systolic phase prolonged. During exposure to high Ca^{2+} saline, slow wave amplitude and resting potential declined. Eventually slow waves were eliminated with the membrane potential remaining depolarized to a value near the peak of the final slow waves. Following

Figure 9. Effects of 10 mM NH_4Cl on slow waves in intact longitudinal muscle. Trace (B) represents 4 min incubation in 10 mM NH_4Cl. Traces (A) and (B) are from the same cell. (Recorded with microelectrodes)

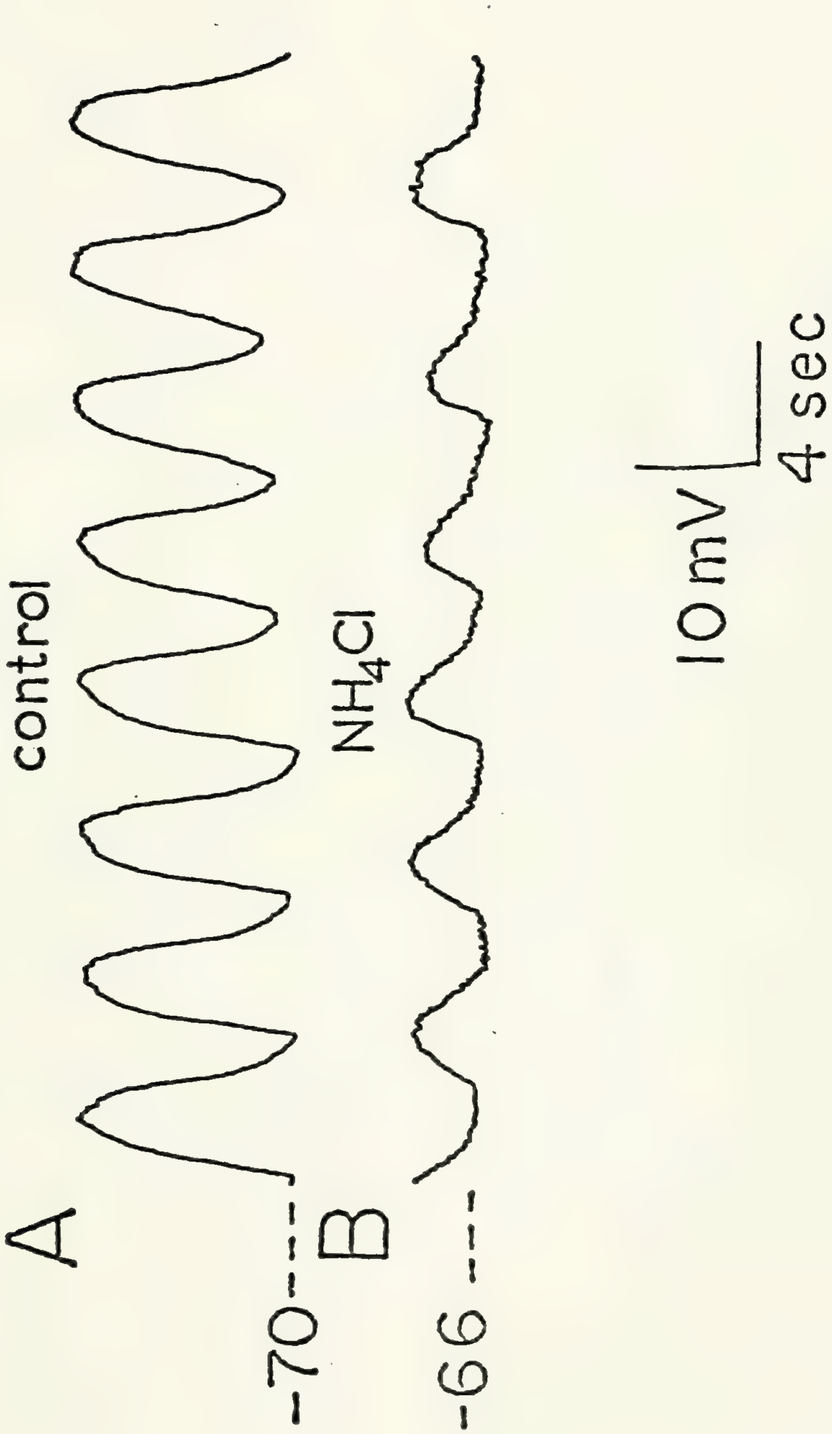

A
-70
control
B
-66
NH_4Cl
10mV
4 sec

Figure 10. Effects of 3 times normal Ca_o^{2+} on slow waves in intact longitudinal muscle. Trace (B) represents 3 min incubation in 3 times normal Ca_o^{2+}. Traces (A) and (B) are from the same cell. (Recorded with microelectrodes).

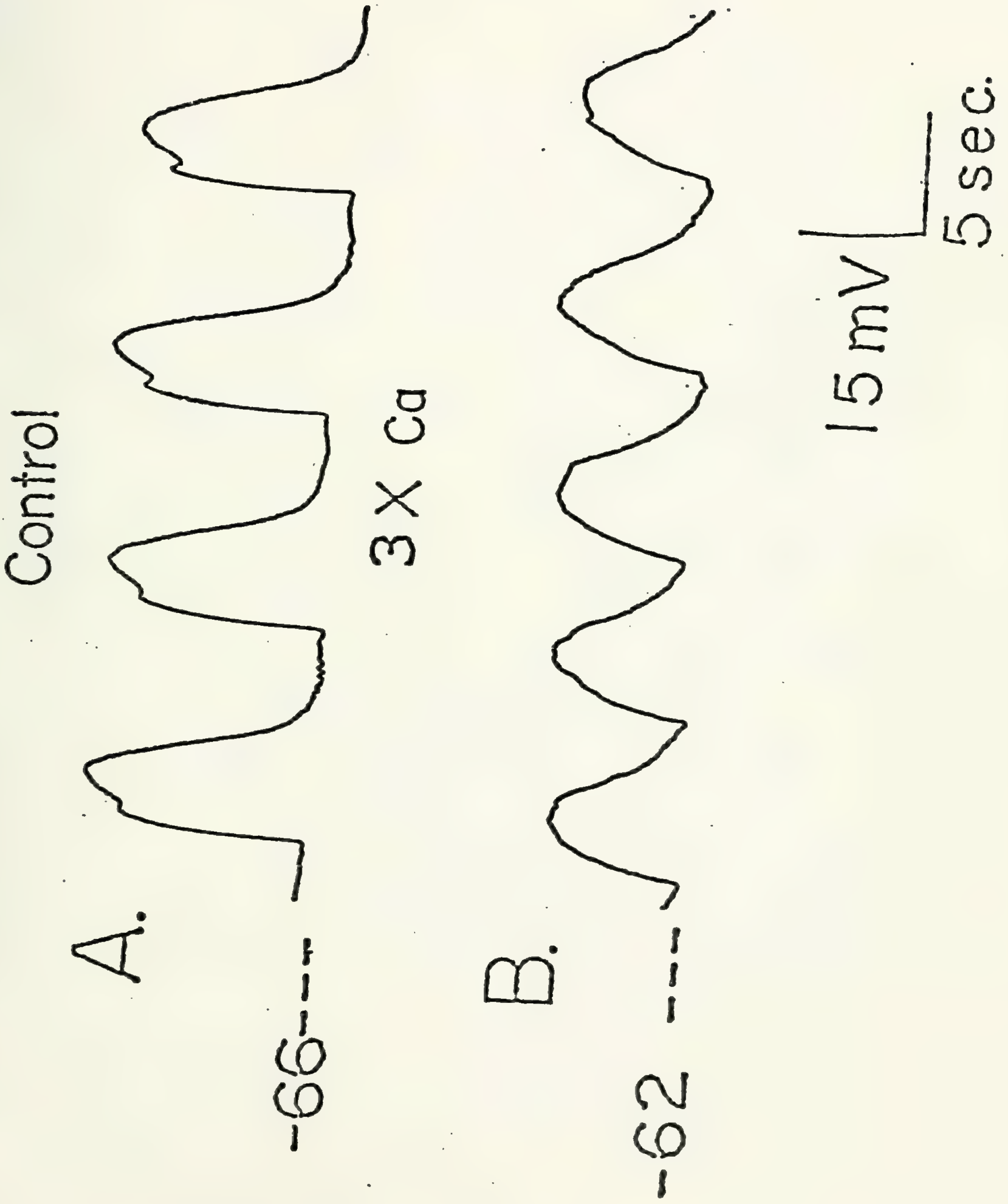
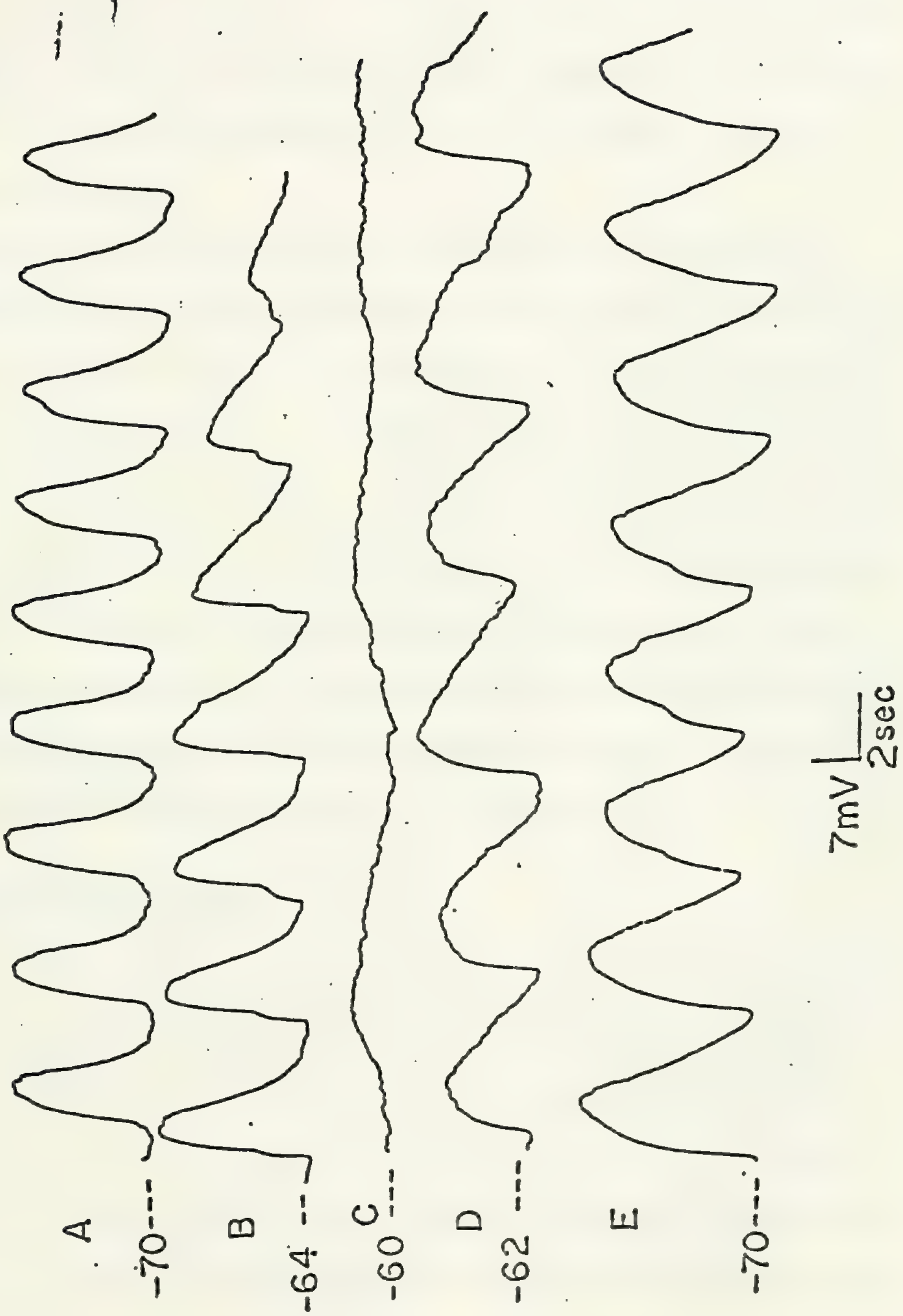

A.
Control
-66
B.
3 X Ca
-62
15 mV
5 sec.

Figure 11. Effects of high Ca_o^{2+} (35 mM) on slow waves in intact longitudinal muscle. (A) Control saline (2.5 mM Ca_o^{2+}); (B) 2 min in 35 mM Ca_o^{2+}; (C) 5 min in 35 mM Ca_o^{2+}; (D) 17 min after return to normal saline; (E) 23 min after return to normal saline. All recordings are from the same cell. (Recorded with microelectrodes)


A
-70
B
-64
C
-60
D
-62
E
-70
7mV
2sec

return to normal saline, slow wave frequency was slower than during initial control conditions (i.e., Figure 11a) and the systolic phase of the slow wave was prolonged. Slow wave frequency gradually approached initial control frequency. In some preparations, there was a transient period of higher frequency on recovery.

Illustrated in Figures 12 and 13 are the effects of 25 mM Ca_o^{2+} on slow waves recorded from isolated longitudinal muscle (n=5). The frequency declined as above with the decline characterized by a prolonged systolic phase. Accompanying this increase in duration of the systolic phase, was a shortening of the diastolic phase. In 4 of the 5 preparations, a decline in slow wave amplitude and small depolarization of the resting potential resulted, however there was no resting potential decline in the fifth preparation. Thus, qualitatively identical effects were observed in isolated and intact longitudinal muscle during exposure to high levels of Ca_o^{2+}, a treatment which might be expected to increase Ca_i^{2+}.

(b) Dibutyryl cAMP (d-cAMP)

Changes in cAMP levels have been reported to produce changes in internal Ca^{2+} levels as well as increases in membrane permeability to Ca^{2+}; cardiac muscle (243,278), molluscan neurons (159), salivary glands (22). It is possible that cAMP might change Ca_i^{2+} levels in smooth muscle also. In Krebs solution of normal tonicity, application of 1-2 mM d-cAMP had no effect on slow waves (n=7). Addition of 1-2 mM d-cAMP in solutions made

Figure 12. Effects of 25 mM Ca_o^{2+} on slow waves in isolated longitudinal muscle. Trace (B) represents 4 min incubation in 25 mM Ca_o^{2+}. Trace (C) is after 10 min in normal saline. Traces (A) and (B) are from the same cell; trace (C) is from a different cell in a nearby region. (Recorded with microelectrodes)

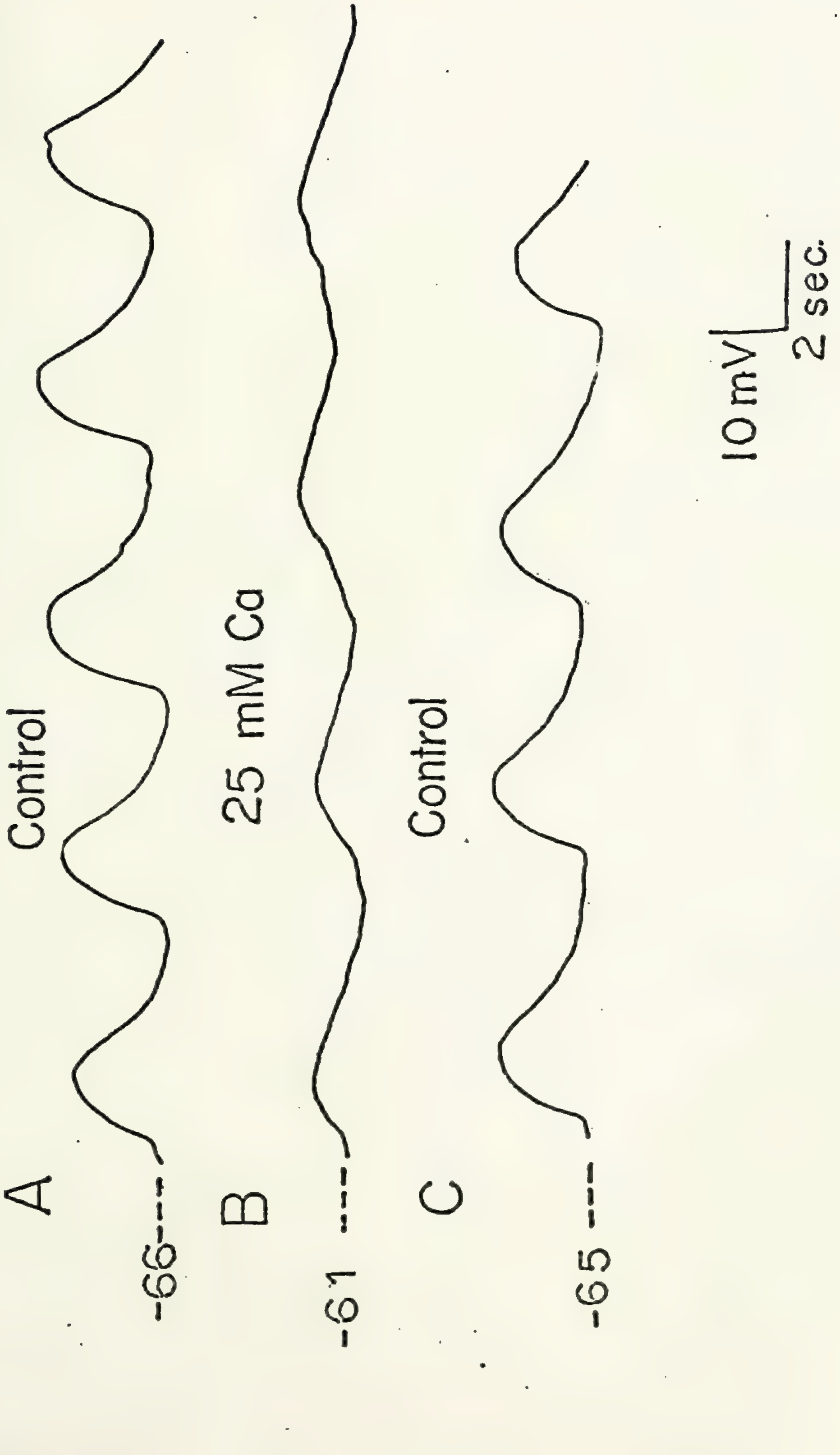

A
-66
Control
B
-61
25 mM Ca
C
-65
Control
10 mV
2 sec.

Figure 13. Effects of 25 mM Ca_o^{2+} on slow waves in isolated longitudinal muscle. Trace (B) represents 5 min incubation in 25 mM Ca_o^{2+}. Traces (A) and (B) are from the same cell. (Recorded with microelectrodes)

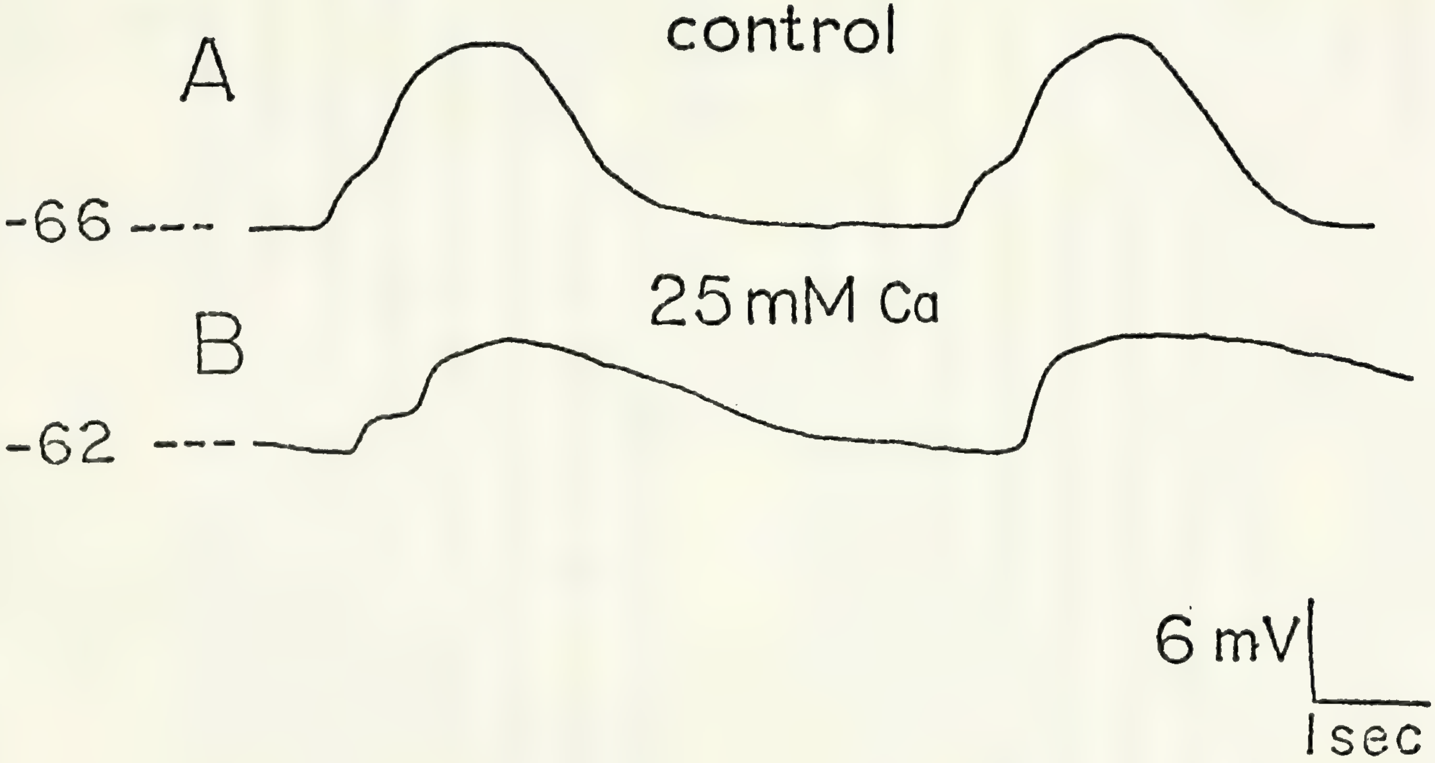

A
control
-66
B
25 mM Ca
-62
6 mV
1 sec

Figure 14. Effects of 2 mM d-cAMP on slow waves in isolated longitudinal muscle. Control and experimental solutions contain 50 grams of sucrose per liter of saline. Trace (B) represents 37 min incubation in 2 mM d-cAMP. Traces (A) and (B) are from the same cell. (Recorded with microelectrodes)

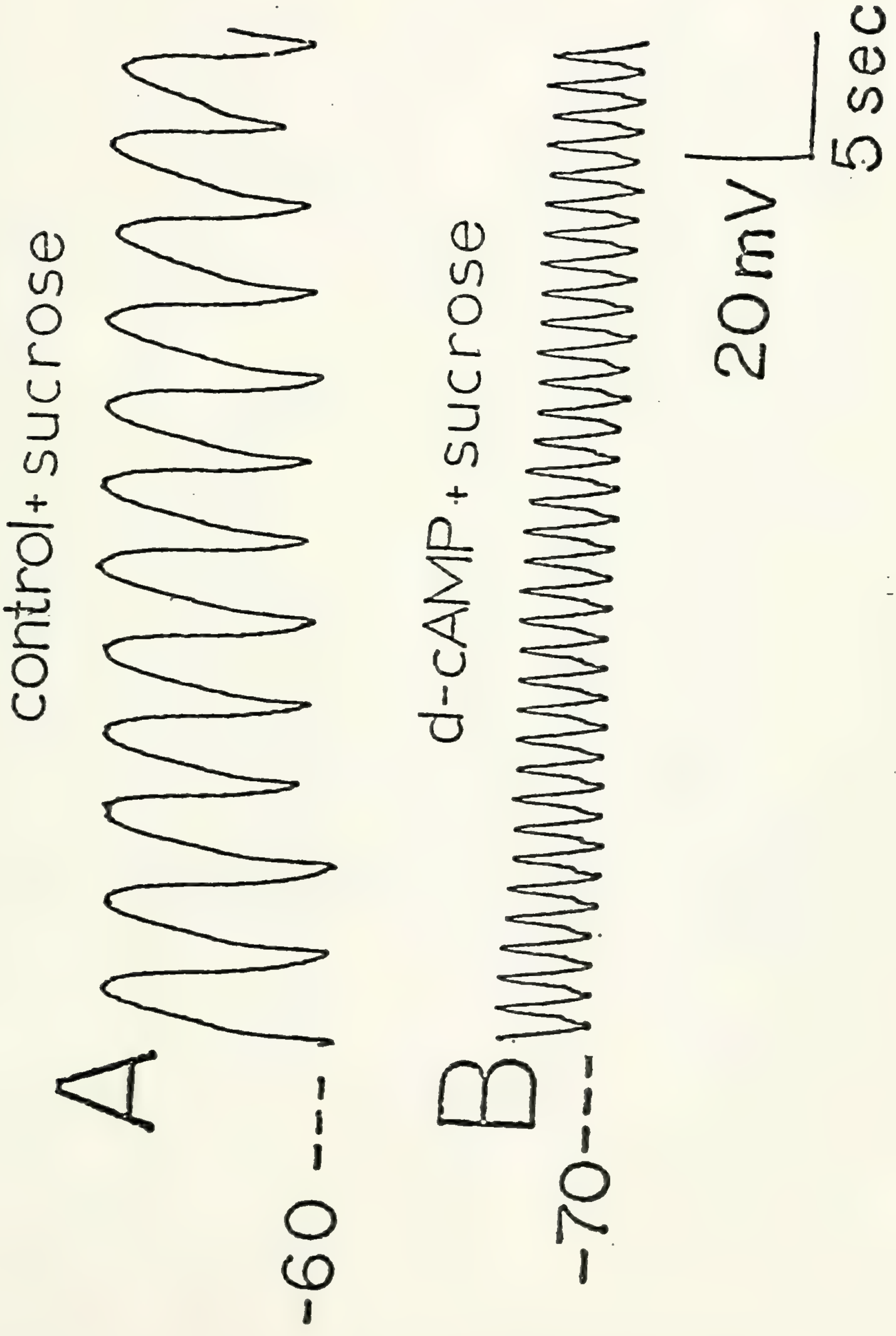
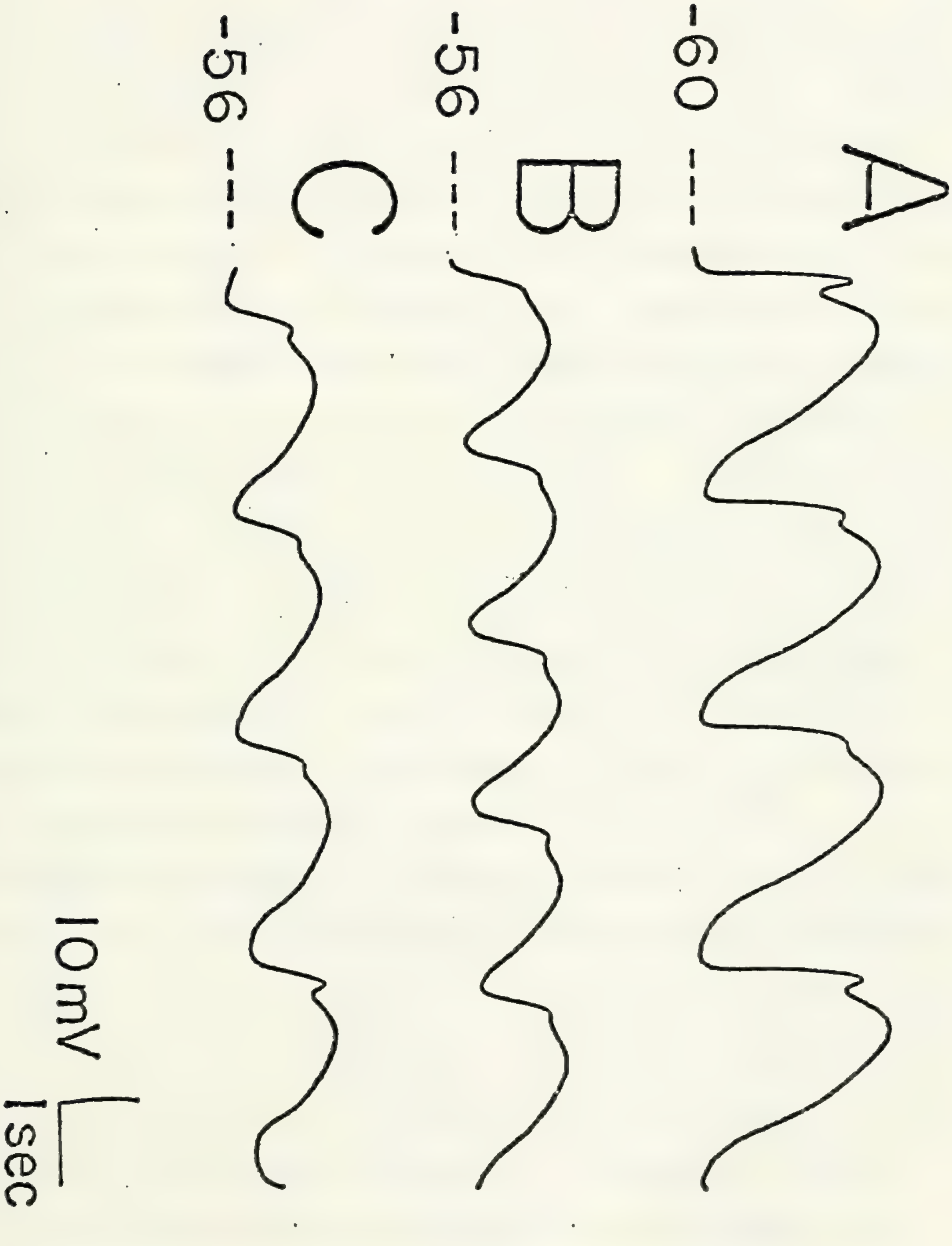

A
control + sucrose
-60
B
d-cAMP + sucrose
-70
20mV
5 sec

Figure 15. Effects of decreasing internal pH on slow waves in intact longitudinal muscle. (A) Control (MOPS) saline aerated with 100% O_2. (B) 1 min after start of aeration with 95% O_2-5% CO_2. (C) 45 sec later. Traces (B) and (C) are from the same cell; trace (A) is from a different cell in a nearby region. (Recorded with microelectrodes)


A
B
C
-60
-56
-56
10mV
1sec

the small size of smooth muscle cells as well as the number of cells which would have to be injected to resolve a light signal (aequorin (124); arsenazo (2)). CTC permeates the plasma membrane and can be incorporated into cell membranes. The fluorescence of CTC is sensitive to Ca^{2+} and Mg^{2+} (60,61,114, 128,212). CTC forms fluorescent complexes with Ca^{2+} and Mg^{2+} in aqueous solution and when biological membranes are added to the solutions CTC fluorescence is enhanced (60,61). Fluorescence titration curves show that the dye exhibits a selectivity for Ca^{2+} over Mg^{2+} (61). Increasing the Ca^{2+} concentration (3 to 14 times) bathing lobster nerves and squid axons produced increases in CTC fluorescence signals during stimulation, while 14 fold changes in Mg^{2+} had no effect on the signals (128). In studies with erythrocyte ghosts (128), carciac muscle cells (114), and rabbit neutrophils (212) CTC fluorescence was sensitive to Ca^{2+} concentration changes. Chandler and Williams (64a) localized by fluorescence microscopy CTC deposits within mitochondria during periods of high Ca^{2+} uptake. The Ca^{2+} and Mg^{2+} complexes of CTC can be spectrally distinguished. In this study I used wavelengths which optimize for the Ca^{2+} complex.

Increases or decreases in Ca_o^{2+} produced corresponding changes in CTC fluorescence levels, i.e., increases in Ca_o^{2+} caused increases in fluorescence. Oscillations in CTC fluorescence were observed when signal averaging techniques were used. The fluorescence oscillations occurred with the same period as slow waves. It was necessary to average between 15-50 slow waves to resolve a fluorescence oscillation. Illustrated in Figures 16 and 17 are simultaneous voltage and fluorescence records gathered from the same region of intact longitudinal muscle. The waveform of

Figure 16. Summed voltage (upper) and fluorescence (lower) signals from intact muscle using a computer of average transients. For this and Figures 17, 18, 19, 20, and 21 preparations were incubated in 5 mM chlorotetracycline (CTC) for 45-75 min. The recording chamber was then flushed with normal saline before averaging. This record is an average of 15 slow waves. (Cal bar: relative gain in arbitrary units: voltage 5×10^4, fluorescence 10^4, 1.5 sec). Voltage is recorded with a tube electrode. The intestine is in its normal configuration, i.e., longitudinal muscle facing outward.

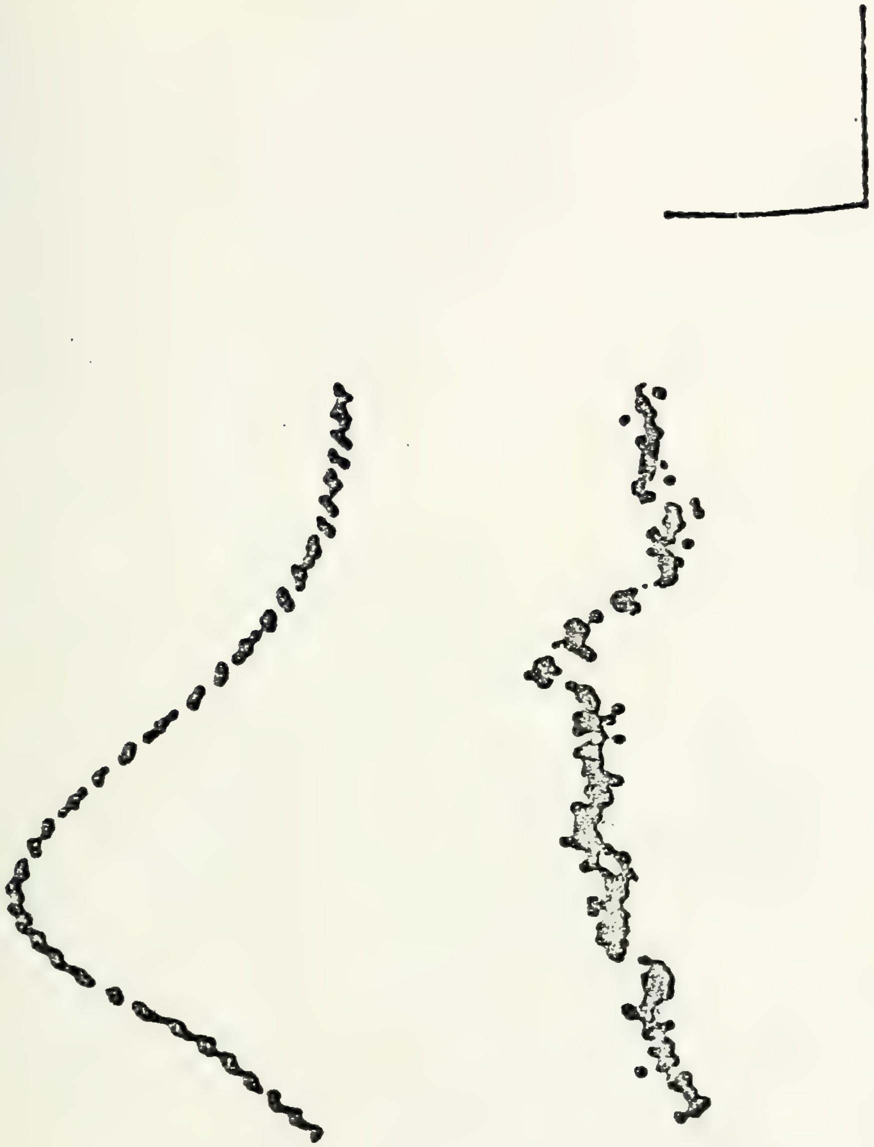
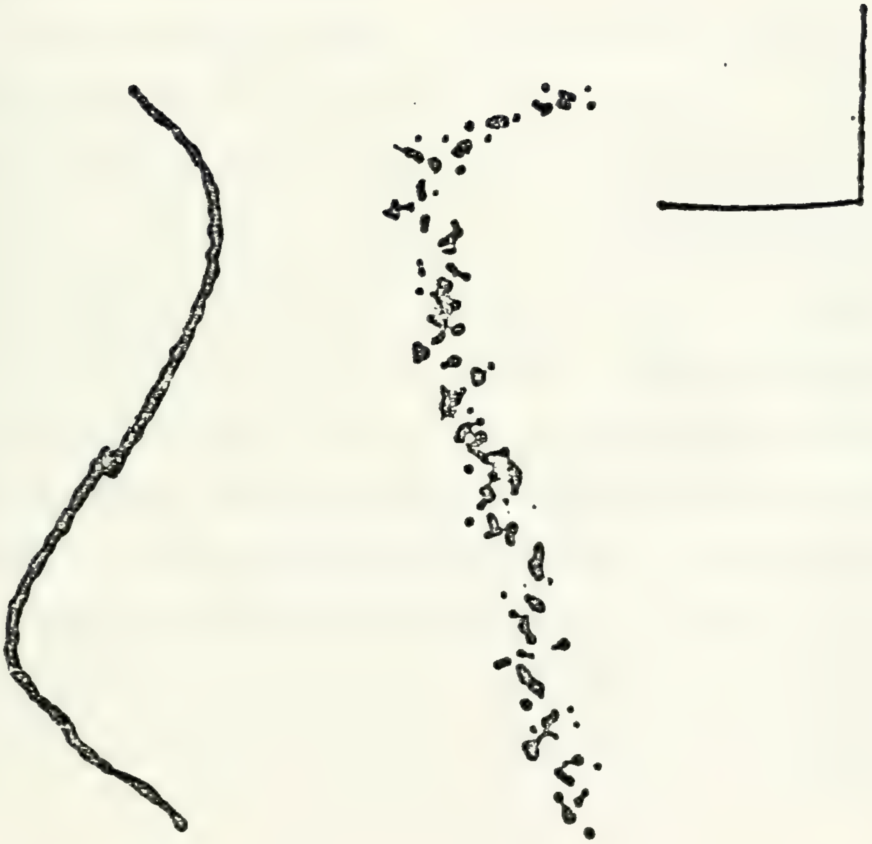


Figure 17. Summed voltage (upper) and fluoresence (lower) signals from intact muscle. This record is an average of 60 slow waves (Cal bar: voltage 5×10^4, fluorescence 10^3, 2 sec). Voltage is recorded with a tube electrode from the intestine in its normal configuration.



the fluorescence signal was ramp-like with the apex of the ramp occurring between the midpoint of the falling phase and the trough of the slow wave.

Fluorescence signals gathered from intact circular muscle, produced more sinusoidal waveforms with the peak of the signal occurring much earlier on the slow wave (Figure 18). In Figures 19 and 20 fluorescence signals gathered from the longitudinal and circular muscle layers of an intact preparation are illustrated.

A possible complication in fluorescence studies arises from light scattering changes due to movement of the preparation. Thus, a preparation displaying oscillatory mechanical activity could produce oscillations in fluorescence signals. To rule out this possibility fluorescence signals were monitored in the absence and in the presence of atropine, an agent which eliminates contractions in cat small intestinal muscle (184). Figure 21 shows fluorescence changes in control saline and following addition of atropine. Oscillations in CTC fluorescence could be observed in the absence of detectable mechanical activity, it can be concluded that the observed oscillations in CTC levels do not result from rhythmic contractile activity.

Figure 18. Summed voltage (upper) and fluorescence (lower two records) signals from intact muscle. This record is an average of 45 slow waves (Cal bar: voltage 5 x 10^4, fluorescence 10^3 and 5 x 10^2, 1.5 sec.) Voltage is recorded with a tube electrode. For this record a mucosa free preparation (also devoid of the muscularis mucosa) was everted and mounted on the tube electrode.

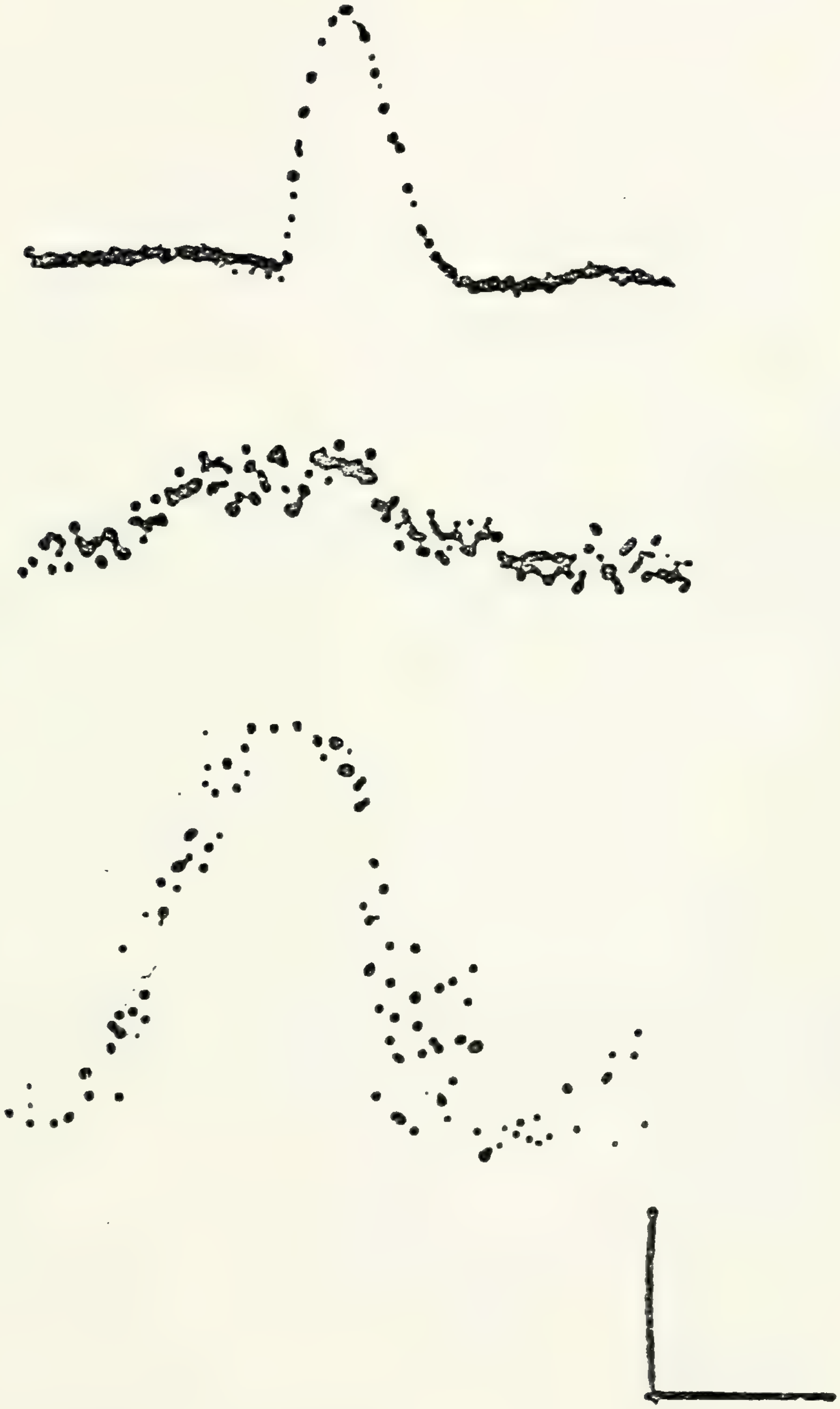


Figure 19. Summed voltage (upper) and fluorescence (lower) signals from intact longitudinal muscle. This record is an average of 40 slow waves (Cal bar: voltage 10^4, fluorescence 5×10^3, 2 sec). Voltage is recorded with a suction electrode. For this record a 2 cm^2 sheet of muscle was used.

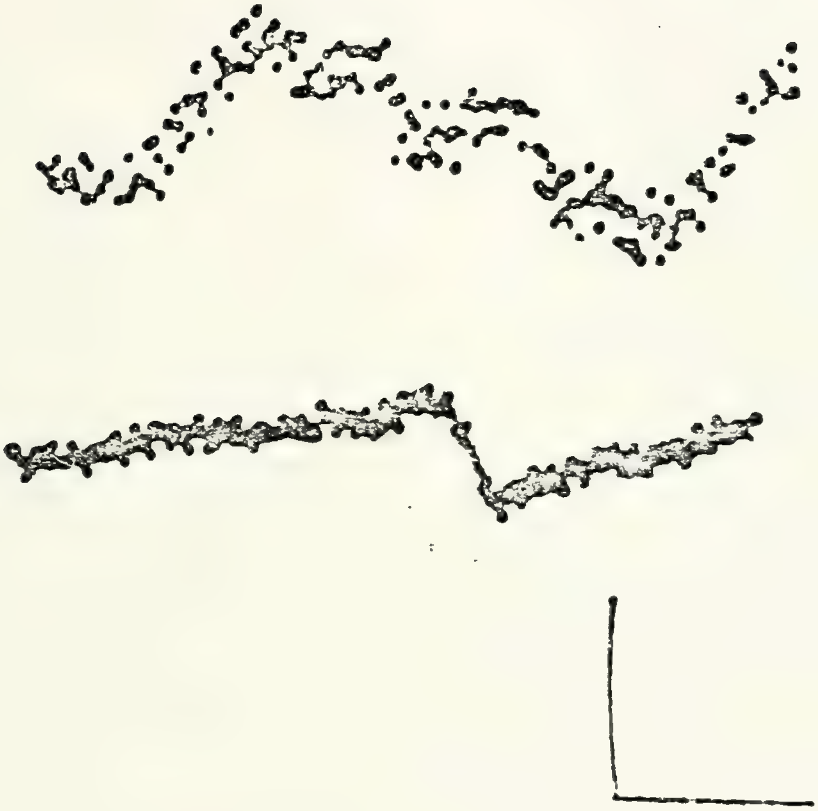


Figure 20. Summed voltage (upper) and fluorescence (lower) signals from intact circular muscle. For this record the muscle sheet used in Figure 19 was inverted. This record is an average of 35 slow waves (cal bar: voltage 5×10^4, fluorescence 10^4, 1.5 sec). Voltage is recorded with a suction electrode.

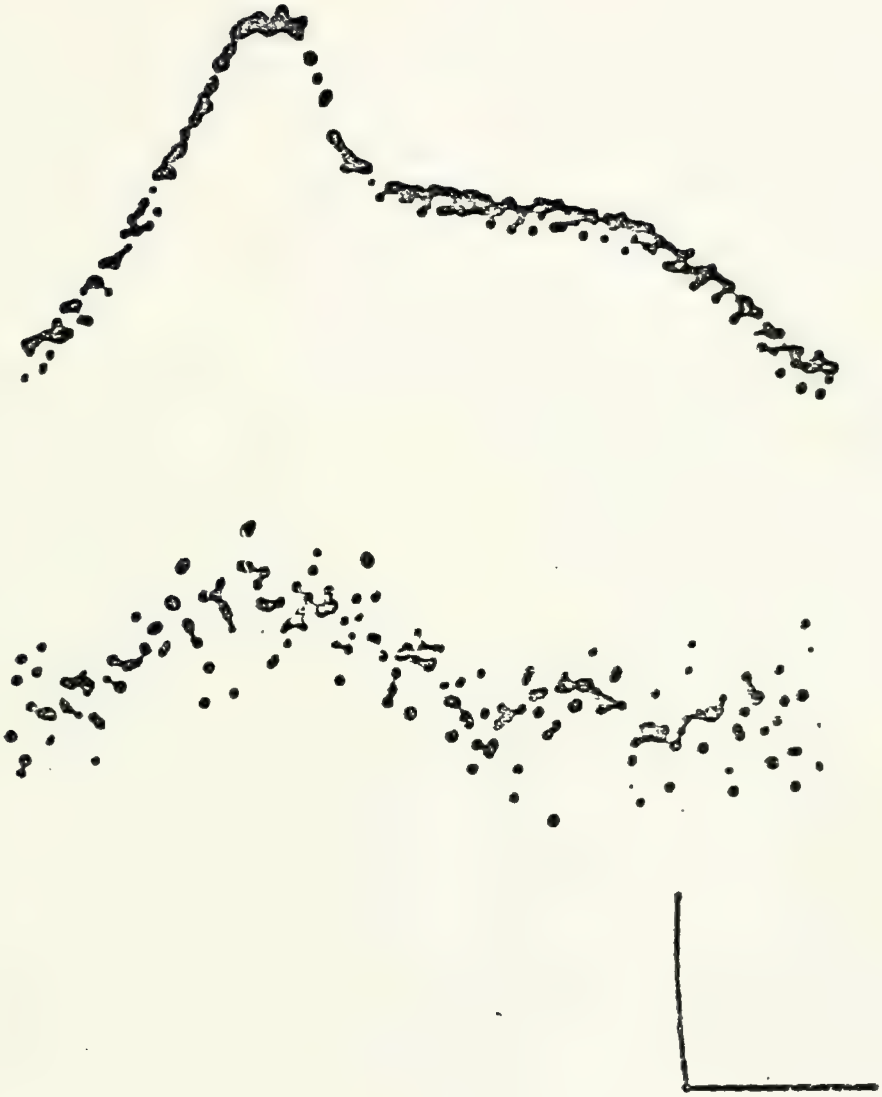
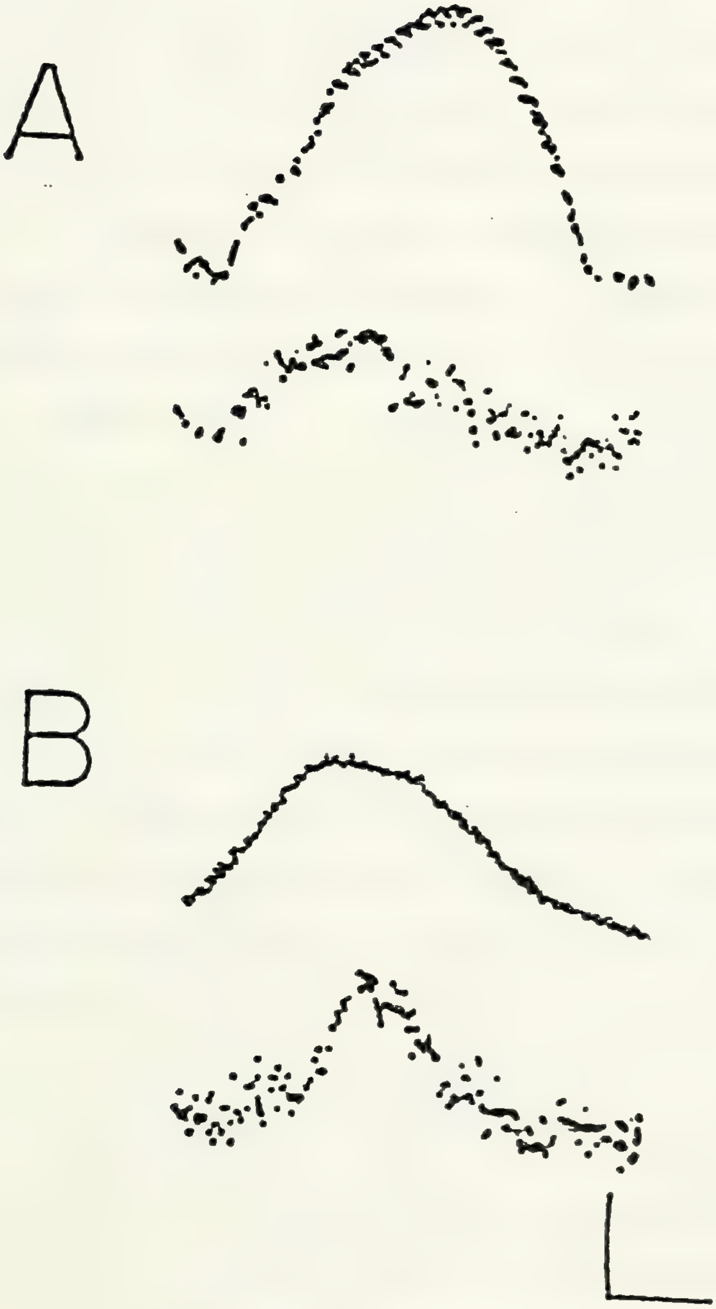


Figure 21. Summed voltage (upper) and fluorescence (lower) signals from intact circular muscle (devoid of the muscularis mucosa). (A) In normal saline. this record is an average of 29 slow waves (cal bar: voltage 10^4, fluorescence 10^3, 1 sec). (B) In the presence of 10^{-4} M atropine. This record is an average of 25 slow waves (cal bar: voltage 10^4, fluorescence 10^3, 1 sec). Voltage is recorded with a suction electrode.


A
B

A. Discussion

Incubation of muscle segments in saline with low Ca^{2+} concentrations decreases slow wave frequency from control levels. The slow waves in low Ca are characterized by a prolonged diastolic phase which decreases in duration following return to normal Ca_o^{2+} levels. Increasing external Ca^{2+} up to 4 times normal levels, produces an increase in slow wave frequency. Further increases in Ca_o^{2+} result in a decrease in slow wave frequency. In either case, increasing external Ca^{2+} results in a prolongation of the systolic phase and reduction in the duration of the diastolic phase of the slow wave compared to control activity. Several lines of evidence suggest that the changes brought about are not due to changes in extracellular Ca^{2+} levels *per se*: (1) treatments which block the influs of Ca^{2+} into cells, without disturbing extracellular Ca^{2+} levels (Ca channel blockers, increasing external Mg^{2+} levels), have identical effects on slow wave frequency as reduction in Ca_o^{2+} levels. (2) Treatments which may alter internal Ca^{2+} levels, (a) increasing or (b) decreasing internal pH, have identical effects on slow wave frequency as (a) reduction or (b) elevation in Ca_o^{2+}. (3) Oscillations in intracellular Ca^{2+} levels as measured by CTC occur with the same period as slow waves.

Changes in extracellular Ca^{2+} levels may produce corresponding changes in the level of intracellular Ca^{2+}. Changes in intracellular Ca^{2+} may, therefore, produce changes in the waveform and frequency of slow waves. Thus, intracellular Ca^{2+} may play a critical role in the pacemaker process responsible for generation of cat small intestinal slow waves.

As discussed previously (see Introduction, pg. 29), cat small intestinal slow waves result from oscillations in the activity of an electrogenic Na pump. Changes in the waveform and frequency of slow waves are reflections

of alteration in the level of electrogenic Na transport. My results are consistent with an inhibitory effect of intracellular Ca^{2+} on Na pump activity. Dunham and Glynn (202) and Epstein and Whitham (110) showed that MgATP, rather than ATP, is the active substrate for the Na pump. Ca_i^{2+} can compete with Mg_i^{2+} for ATP binding, thus reducing Na pump activity. Schön _et_ _al_. (268) postulated that Ca_i^{2+} may inhibit Na pump activity by competing with intracellular Na^+ for binding sites on the ATPase molecule and several investigators (56,176,254) have shown that intracellular Ca^{2+} decreases mitochondrial ATP production which will result in a reduction in Na pump activity. This last possibility will be discussed later. Increases in intracellular Ca^{2+} levels produce a prolongation of the slow wave systolic phase, the phase of the slow wave associated with low levels electrogenic Na transport. Reductions in intracellular Ca^{2+} concentration cause a prolongation of the slow wave diastolic phase, the period when electrogenic Na transport is at a high level. As discussed on page 32, the effects of alterations in Ca^{2+} levels on cat small intestine are not consistent with the Tomita and Watanabe (288) hypothesis of slow wave generation.

Oscillations in the level of Ca_i^{2+} near the plasma membrane could produce oscillations in the activity of Na-K ATPase and therefore membrane potential. Since the frequency of oscillations in NADH levels is Ca sensitive (see Section IIIc), the slow wave generation system may be coupled, via a Ca dependent process, to the NADH/NAD redox system.

The CTC studies indicate that an oscillation in intracellular Ca^{2+} occurs during the course of the slow wave. The exact source of this Ca_i^{2+} is not clear. Hallett _et_ _al_. (128) suggested that CTC monitors Ca^{2+} changes

in the immediate vicinity of the inner surface of the plasma membrane. Others (60,61,64a) have concluded that CTC also binds to the membranes of various intracellular organelles, although a large proportion of the CTC is bound to the plasma membrane. Since in the immediate vicinity of a membrane, bound and free Ca^{2+} are in equilibrium, a change in the level of bound Ca^{2+} should, therefore, reflect a corresponding change in Ca_i^{2+}.

The spontaneous slow waves of mammalian small intestine exhibit a frequency gradient in the oral to aboral direction (9). The basis for the slow wave frequency gradient has been attributed to a metabolic gradient down the intestine (29). Regions with faster slow wave frequency display a higher metabolic rate than regions with slower frequency.

An alternative hypothesis, which is supported by the data of Job and Bloomquist (153) who demonstrated a gradient of total Ca in the longitudinal muscle layer of cat small intestine (between 2.46 to 4.27 mM Ca), is that there is a Ca_i^{2+} gradient along the intestine. Job and Bloomquist (153) found duodenal Ca content to be greater than either jejenal or ileal levels. Ileal Ca concentration was smaller than that found in the jejenum. If the gradient of total Ca reflects different levels of intracellular ionized Ca, then this may account for the slow wave frequency gradient; since, the frequency of slow waves is sensitive to the level of intracellular ionized Ca. The ileal region, characterized by low Ca levels, demonstrates low frequency slow waves with prolonged diastolic phases, as compared with the duodenum where Ca levels are higher. This correlates well with the experimental effects of altering Ca_i^{2+} levels.

This latter hypothesis is favorable to the metabolic gradient hypothesis since, following metabolic inhibitors slow wave amplitude not

frequency is predominantly affected (152,230). If a metabolic gradient is controlling the frequency of slow waves, then alterations in the metabolic state of the tissue should result in a change in the frequency of slow waves. Since slow wave frequency is not changed by metabolic inhibitors, a metabolic gradient is probably not controlling the gradient of slow waves along the intestine. However, the metabolic gradient may be produced by a gradient of slow waves (and therefore contractions) along the intestine.

An as of yet unexplained phenomena is the voltage sensitivity of slow waves (81). Application of hyperpolarizing current decreases slow wave frequency, while depolarizing currents increase frequency. Depolarization of the intestinal membrane leads to an increased influx of Ca^{2+} (178,301). The increased influx of Ca^{2+} could produce an acceleration in slow wave frequency as is observed following small increases in Ca_i^{2+} (i.e., up to 4 times increases in Ca_o^{2+}, or by decreasing internal pH).

B. Possible Involvement of Intracellular Ca Compartments in Generation of Contraction in Smooth Muscle

Summary

(1) Voltage dependent release of intracellular Ca, without an accompanying influx of Ca, was demonstrated.

(2) Intracellular Ca compartments may be partitioned away from the plasma membrane.

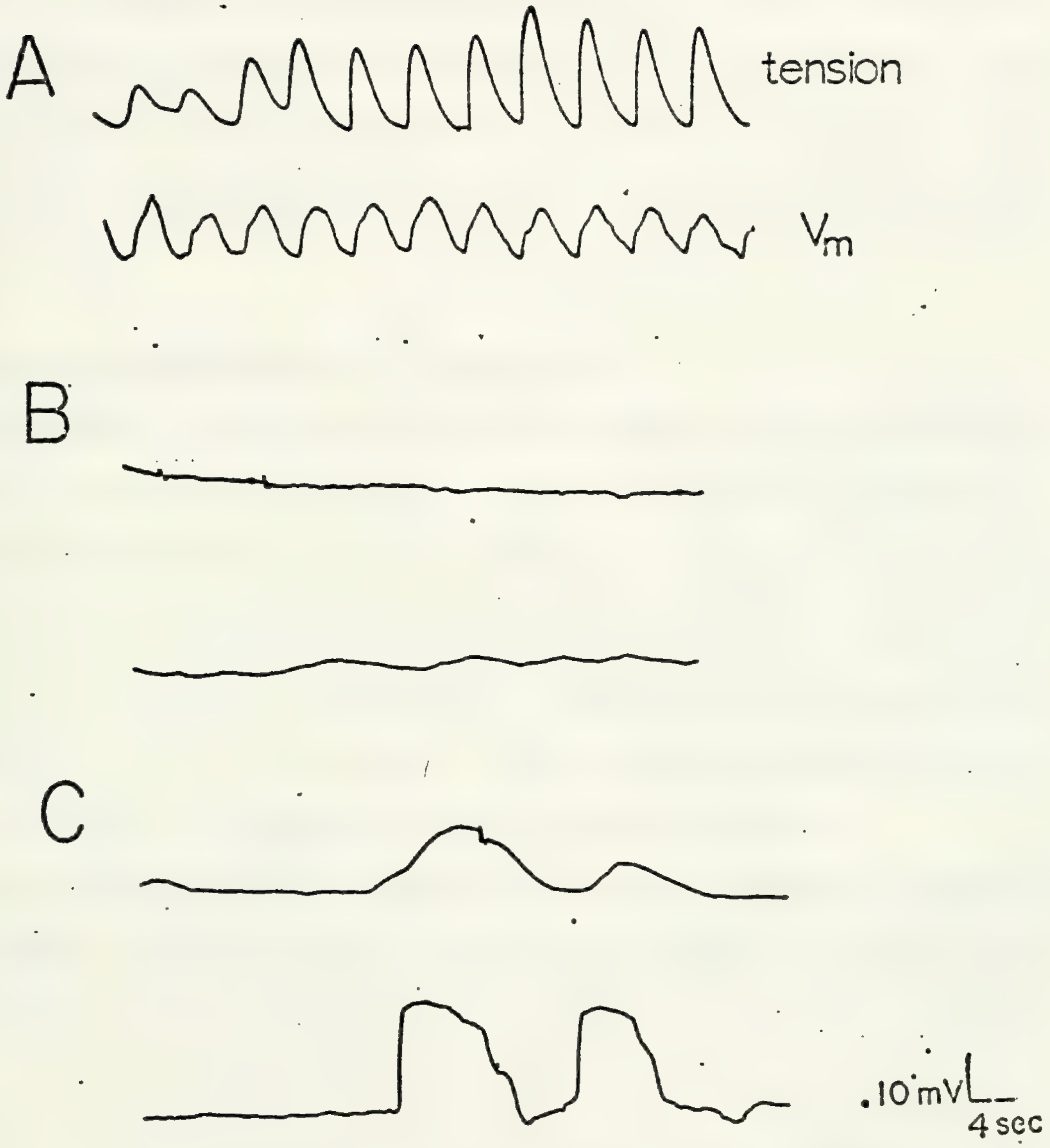
B. Possible Involvement of Intracellular Ca Compartments in Generation of Contractions in Smooth Muscle

(i) Spontaneous Activity in Ca^{2+} Free Saline

During incubation in Ca^{2+} free saline (with EGTA; see Methods) normal electrical and mechanical activity of the small intestine ceases (see Figure 1). However, following a period of quiescence, lasting 1-45 min, spontaneous electrical and mechanical activity reappeared in 30% (n=50) of the muscle segments studied in this series of experiments (see Figure 1c). Rhythmic electrical activity in Ca^{2+} free saline similar to that of Figure 1 has been previously described by Prosser *et al.* (234) who showed that the rhythmic voltage changes (prolonged potentials) result from Na ions traversing channels normally used by Ca^{2+}. Rhythmic contractions accompanied prolonged potentials in 80% of the spontaneous preparations (see Figure 1c). The mean amplitude of the contractions associated with the prolonged potentials was 60% of control amplitude, although some contractions were as large as 5 times control activity. Spontaneous electrical and mechanical activity in Ca^{2+} free saline persisted for periods greater than 1 hour in some preparations.

In Ca^{2+} free saline it is unlikely that there is an appreciable inward-directed Ca^{2+} gradient. Therefore, the ability of prolonged potentials to induce contractions suggests that an influx of Ca^{2+} is not a prerequisite for contractions to occur and that internal stores of Ca^{2+} which may be released by rhythmic membrane depolarization can support rhythmic contractile activity. This finding is in marked contrast to several other studies in which depolarization induced release of internally stored Ca^{2+} required an accompanying influx of Ca^{2+} from the external saline (5,59,94,95,142).

Figure 1. (A) Pressure electrode recording of electrical activity (V_m, lower trace) and corresponding mechanical activity (upper trace) in control saline solution. (B) Resulting activity following incubation for 7 min in Ca^{2+} free saline. (C) Exposure for 40 min to Ca^{2+} free saline. Recordings are from intact cat longitudinal muscle.


A
tension
Vm
B
C
.10 mV
4 sec

To exclude the possibility that Ca might be leeched from the mucosa into the interstial fluid and act as an extracellular Ca^{2+} store to the muscle cell, 30 preparations in which the mucosa was removed were tested. The results of incubation of mucosa-free preparations in Ca^{2+} free saline were qualitatively the same as those obtained for whole preparations except the success rate of obtaining EGTA-induced activity was diminished in preparations devoid of the mucosa, possibly due to the more extensive manipulation of the tissue.

(ii) Triggered Contractions in Ca^{2+} Free Saline

Since the bulk of the preparations which remained mechanically quiescent in Ca^{2+} free saline were also electrically quiescent I attempted to evoke contractile activity in these preparations by inducing membrane potential changes. Contractions, such as those shown in Figure 2, were elicited by electrical stimulation to preparations remaining otherwise quiescent in Ca^{2+} free saline (n=5). In some cases these preparations developed spontaneous electrical and mechanical activity after stimulation.

Following $BaCl_2$ application rhythmic spikes are produced by smooth muscle membranes (301). If the hypothesis that rhythmic membrane depolarization produces rhythmic contractile activity by release of internally stored Ca^{2+} is correct, then addition of $BaCl_2$ to preparations remaining quiescent in Ca^{2+} free saline should generate rhythmic membrane depolarizations with accompanying contractions. Application of $BaCl_2$ elicited rhythmic spikes with contractions (Figure 3) in preparations remaining quiescent in Ca^{2+} free saline. Activity could be evoked even after preparations had been in Ca^{2+} free saline for over 2 hours.

Figure 2. Application of electrical stimulation to a segment of mucosa-free intact longitudinal muscle remaining quiescent after 30 min exposure to Ca^{2+} free saline.

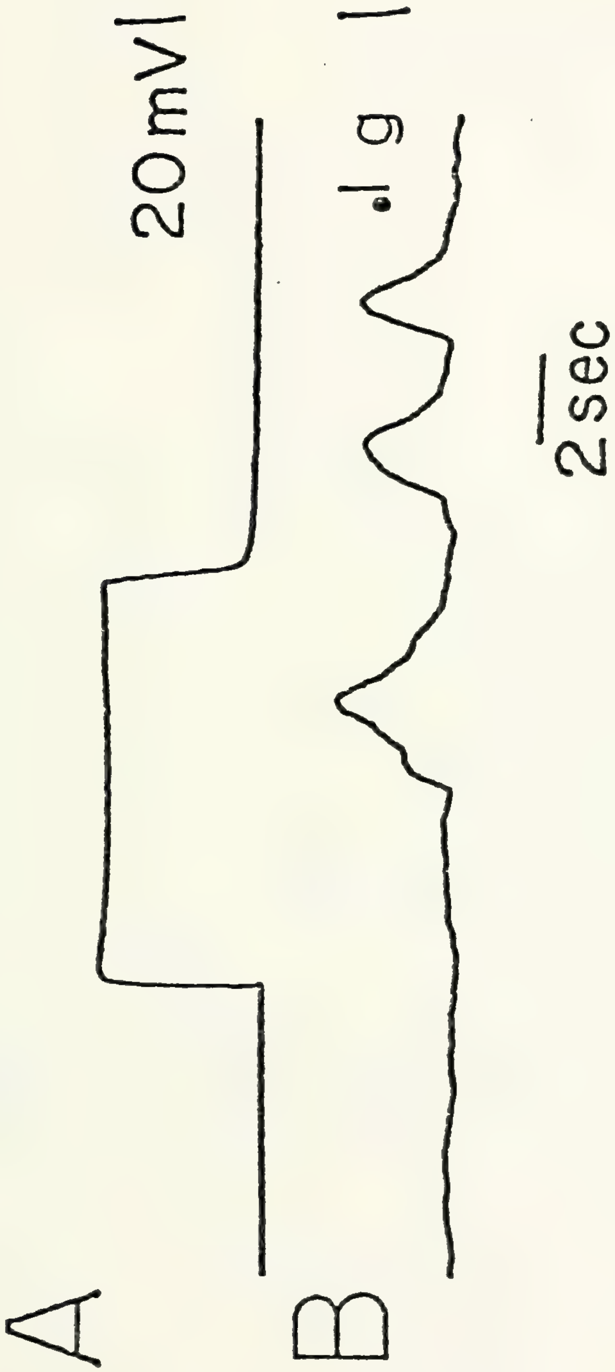
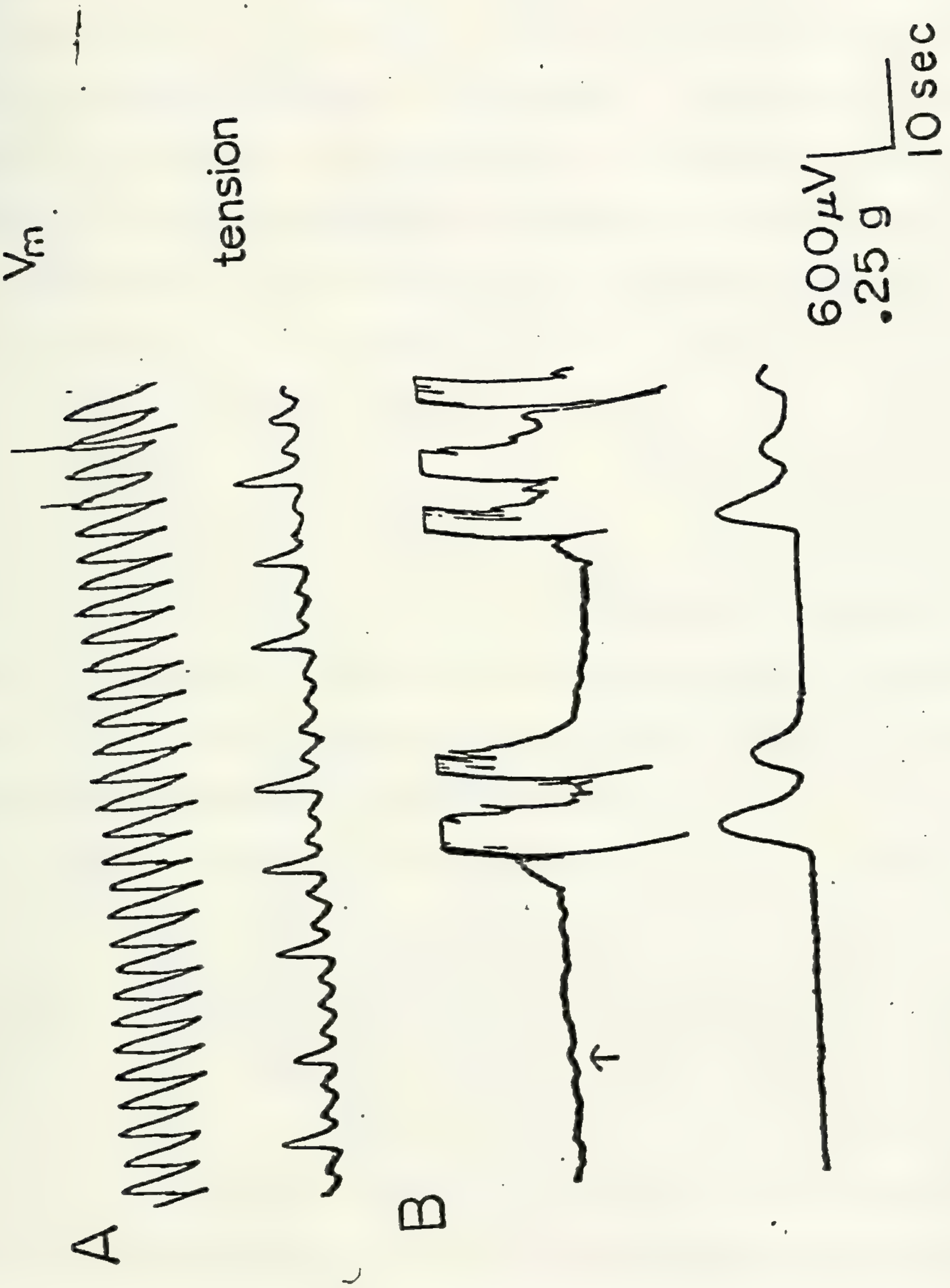

A
B
20mV
1 g
2 sec

Figure 3. (A) Tube electrode recording of electrical (V_m, upper trace) and corresponding mechanical activity (lower trace) in control saline from a mucosa-free segment of intact cat small intestine in the normal configuration. (B) 30 sec (at arrow) after the start of 3 mM $BaCl_2$ in Ca^{2+} free saline; the preparation was incubated in Ca^{2+} free saline for 175 min.


A
B
V_m
tension
600μV
.25 g
10sec

Spontaneous electrical and/or mechanical activity was not observed in segments of either isolated longitudinal or isolated circular muscle during incubation in Ca^{2+} free saline. However, mechanical activity was evoked following depolarization of the membrane. Application of depolarizing current (n=3) (Figure 4) or BaCl addition (n=2) induced contractions in segments of isolated longitudinal muscle during incubation in Ca^{2+} free saline. The preparation illustrated in Figure 4 had been incubated in Ca^{2+} free saline for 25 min. BaCl addition (n=4) to segments of isolated circular muscle induced rhythmic spikes and contractions, even after 50 min incubations in Ca^{2+} free saline (Figure 5). The large difference in the frequency of the electrical and mechanical activity in the preparation illustrated in Figure 5 can be attributed to the large separation distance of the transducer and electrical recording electrode (approximately 15 mm) in this case. It is likely that Ba-induced electrical activity is not synchronized over large segments of isolated circular muscle.

Depolarization of the plasma membrane by K^+ (n=5) or ACh (n=4) also induced contractions in preparations remaining quiescent in Ca^{2+} free saline. Replacement of NaCl with KCl in the bathing saline (to 148 mM) produced phasic contractions (Figure 6). The K^+ induced contractions were approximately twice as large as in normal Krebs. The lower record of Figure 6 demonstrates small rhtyhmic contractions which persisted even after excess KCl was removed (to 5.9 mM K^+_o) (this occurred in 2 of 2 preparations showing rhythmic activity after K depolarization). Following the

Figure 4. Application of electrical stimulation to a segment of isolated longitudinal muscle remaining quiescent after 25 min exposure to Ca^{2+} free saline.

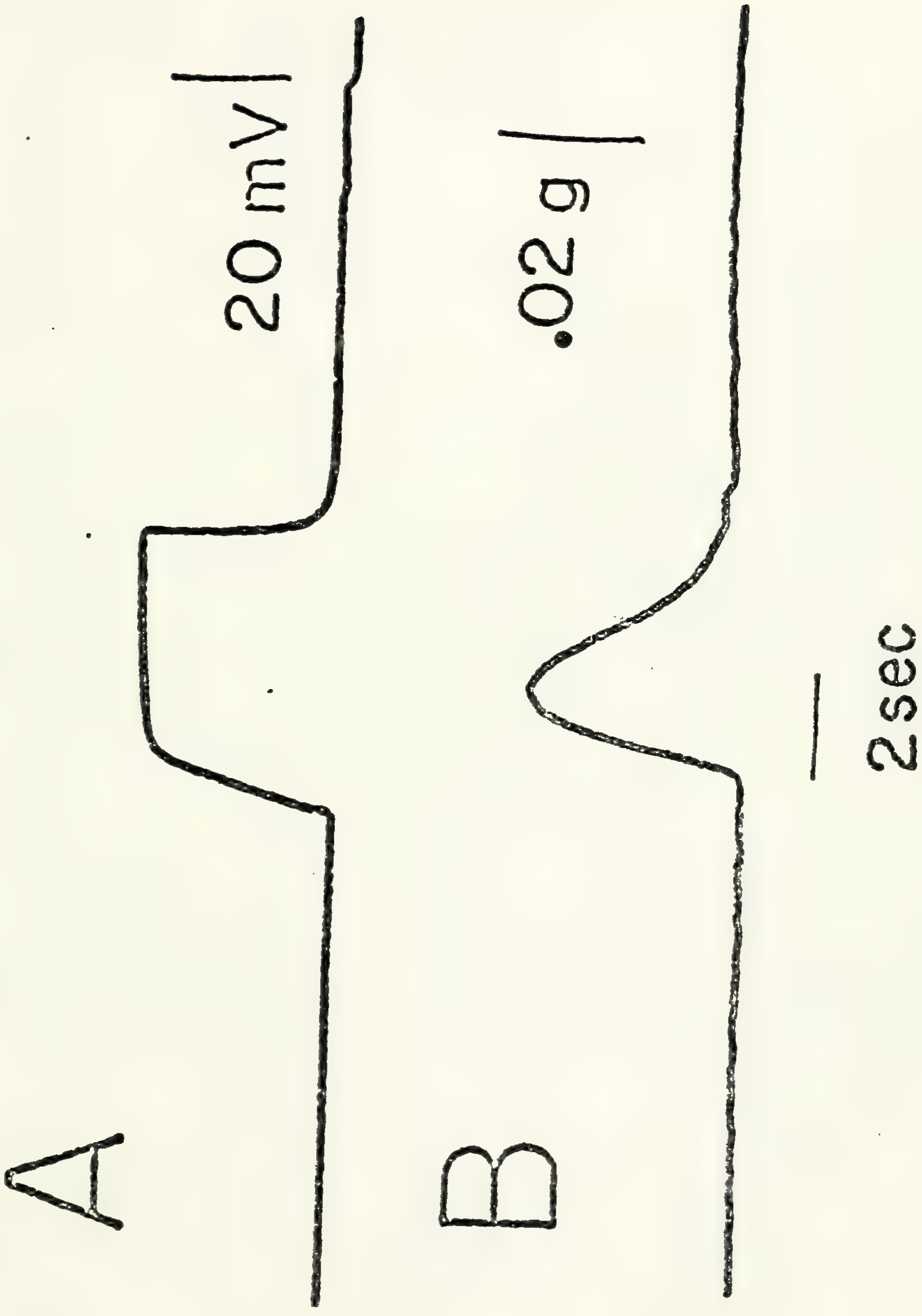

A
B
20 mV
.02 g
2sec

Figure 5. Tube electrode recording of electrical (V_m, lower trace) and corresponding mechanical (upper trace) activity in isolated circular muscle after 50 min incubation in Ca^{2+} free saline. 1 mM Ba^{2+} was added after 45 min in Ca^{2+} free saline.

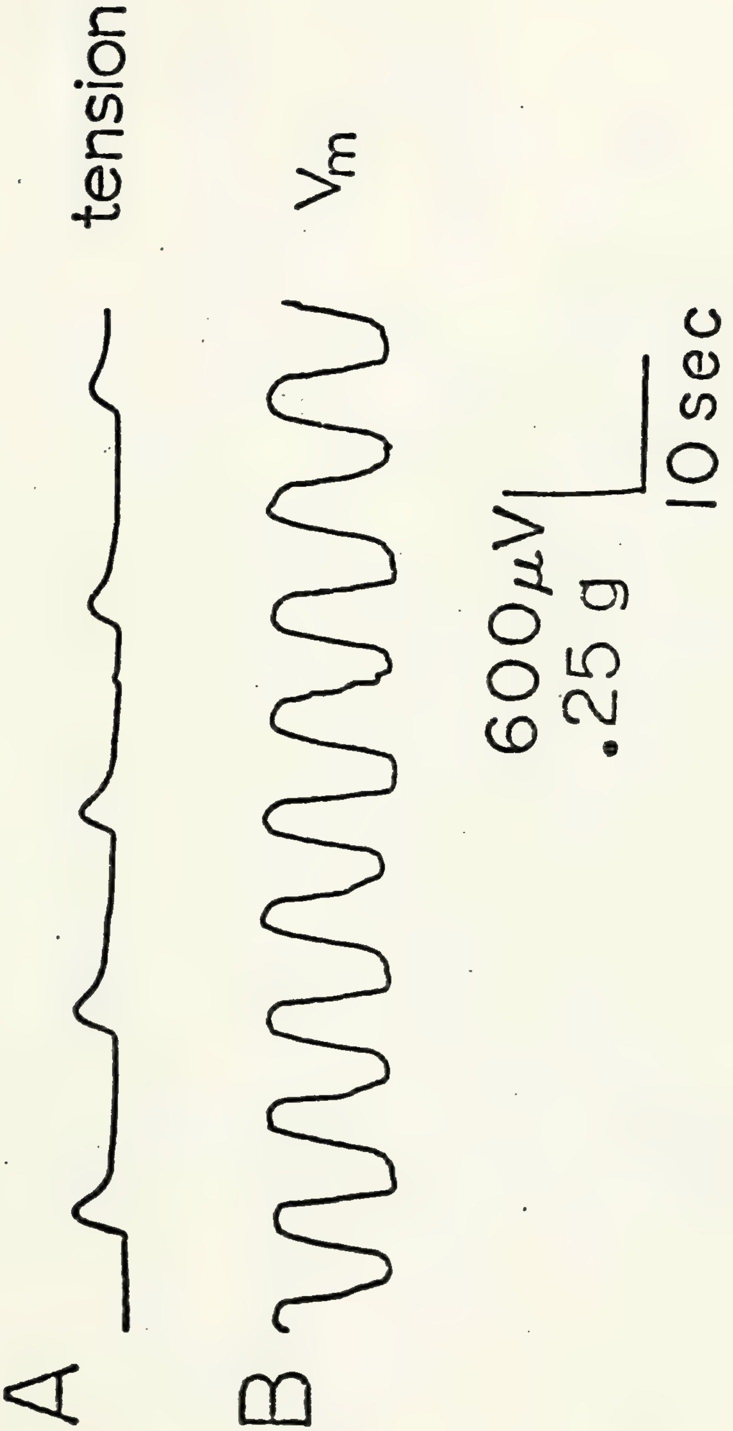
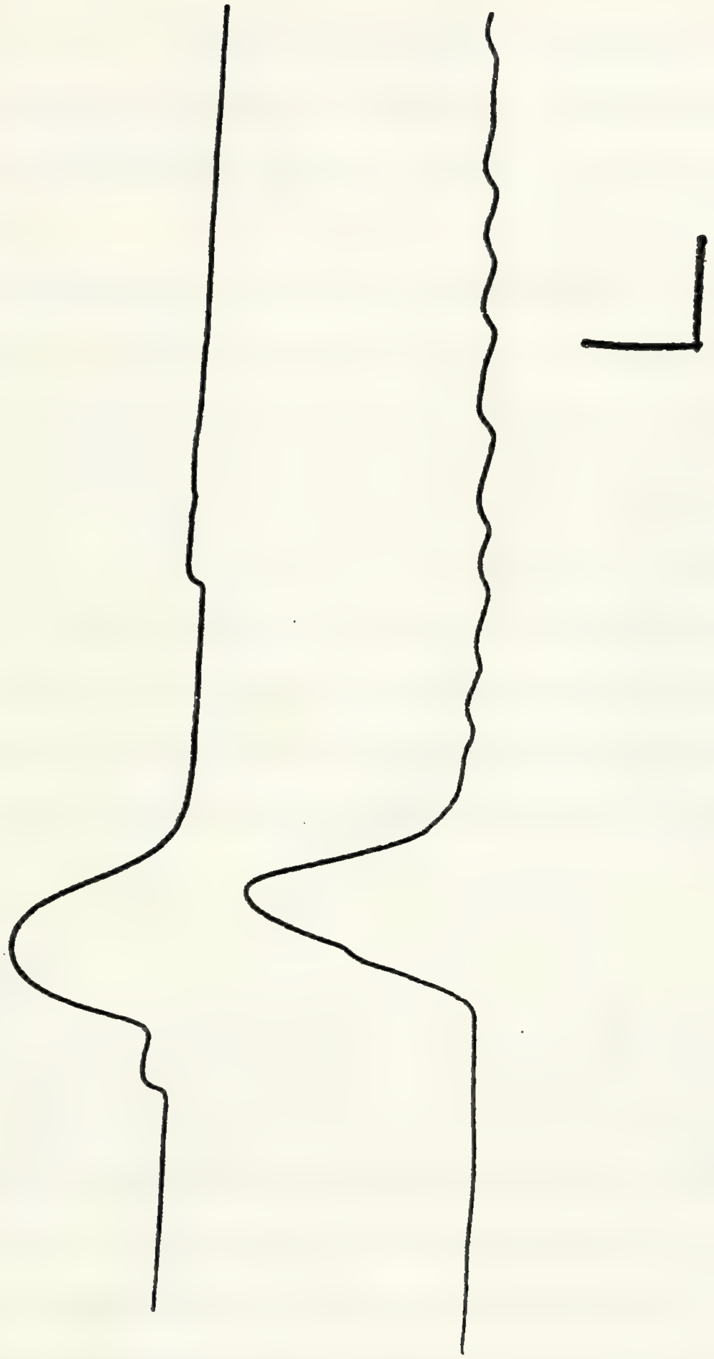

A
tension
B
V_m
600μV
.25 g
10sec

Figure 6. Effects of K^+ depolarization on 2 segments of intact cat intestine remaining mechanically quiescent in Ca^{2+} free saline. In both cases, saline NaCl was replaced by KCl. The preparation in the upper record was incubated in Ca^{2+} free saline for 10 min before exposure to high K^+; lower preparation for 15 min. (cal bar: .2 gm, 7 sec)



addition of 10^{-4} M ACh and 10^{-4} M eserine, rhythmic contractions were produced in otherwise quiescent preparations (Figure 7). ACh application to preparations displaying spontaneous activity in Ca^{2+} free saline enhanced contraction amplitude.

To eliminate the possible presence of an extracellular Ca compartment not removed by incubation in Ca^{2+} free saline, segments of cat small intestine were incubated in saline with no added Ca^{2+} plus 40-45 mM EGTA (n=4). Contractions occurred either spontaneously or could be induced by electrical stimulation (Figure 8) or BaCl (Figure 9) for the first 10 min in high EGTA. After a series of contractions the muscle tone increased and did not relax but showed a slow, steady increase, indicating that the muscle may have gone into contracture. This state was observed for periods up to 20 min and following return to normal saline normal slow waves and contractions resumed.

(iii) Other Preparations

Mechanical activity in EGTA saline was also observed in a number of other smooth muscle preparations. Contractions either occurred spontaneously or were induced by electrical stimulation or $BaCl_2$. Illustrated in Figures 10, 11, 12, and 13 are recordings from cat vena cava (n=2), stomach (n=4), colon (n=3) and esophagus (n=3) during incubation in Ca^{2+} free saline. Spontaneously occurring contractions in the absence of extracellular Ca^{2+} are illustrated in the vena cava (Figure 10) and stomach (Figure 11). The contractions recorded from the colon (Figure 12) were induced by electrical stimulation and those in the esophagus by $BaCl_2$ (Figure 13). Identical types of responses, during incubation in Ca^{2+} free saline, were also observed in cat bladder (n=2) and uterus (n=3).

Figure 7. (A) Segment of intact cat small intestine remaining mechanically quiescent in Ca^{2+} free saline for 15 min. (B) Activity resulting from addition of 10^{-4} M eserine and 10^{-4} M ACh.

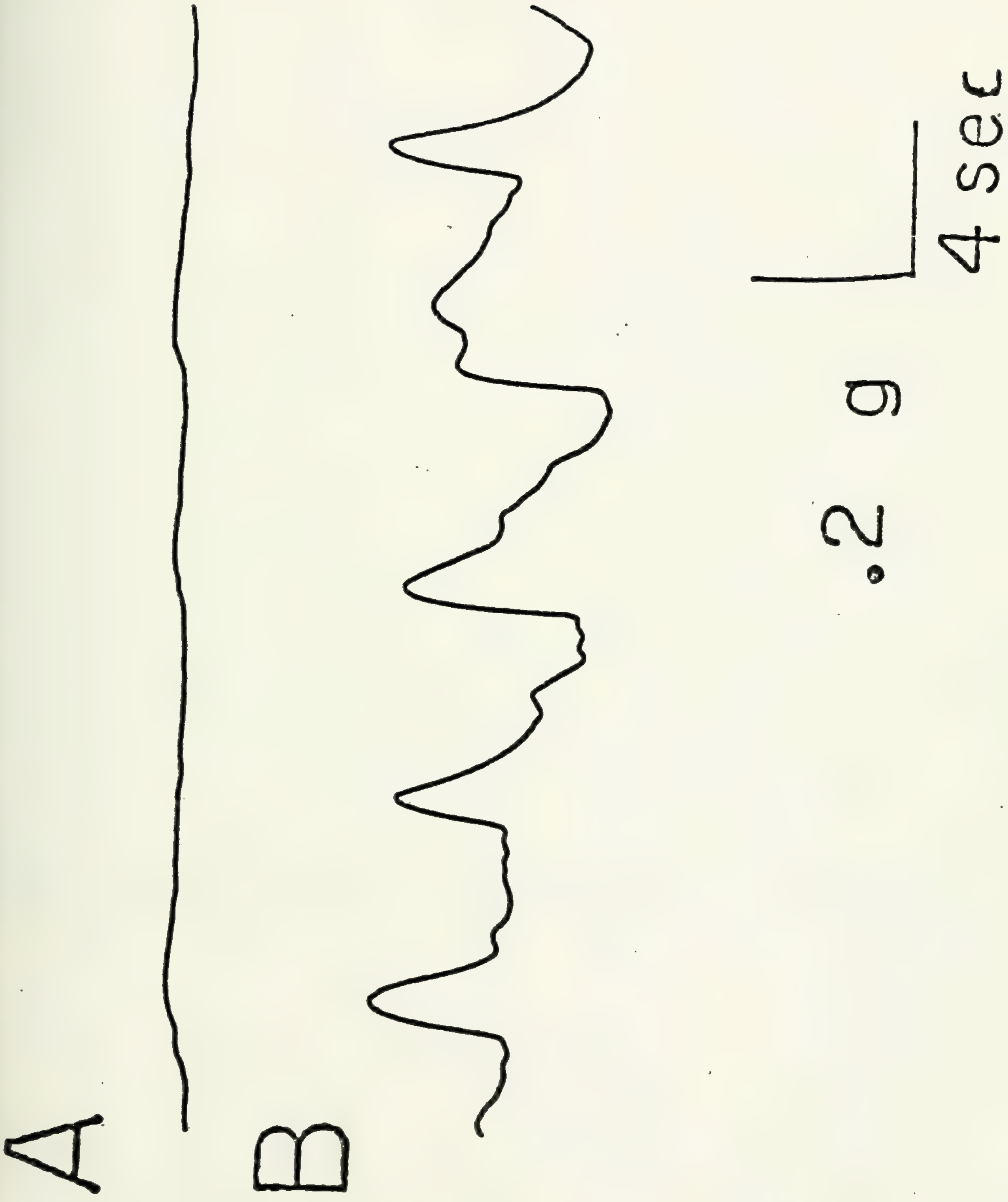

A
B
.2 g
4 sec

Figure 8. (A) Tube electrode recording of electrical (V_m, upper trace) and corresponding mechanical (lower trace) activity in intact cat intestine following incubation in Ca^{2+} free saline containing 1 mM BaCl for 20 min. (B) Corresponding activity following incubation in high EGTA saline (35 mM EGTA added to 0 Ca^{2+} saline) with 1 mM BaCl. Rhythmic contractions could be elicited for 10 min in high EGTA. Slash represents 7 min. Intestine is in its normal configuration.

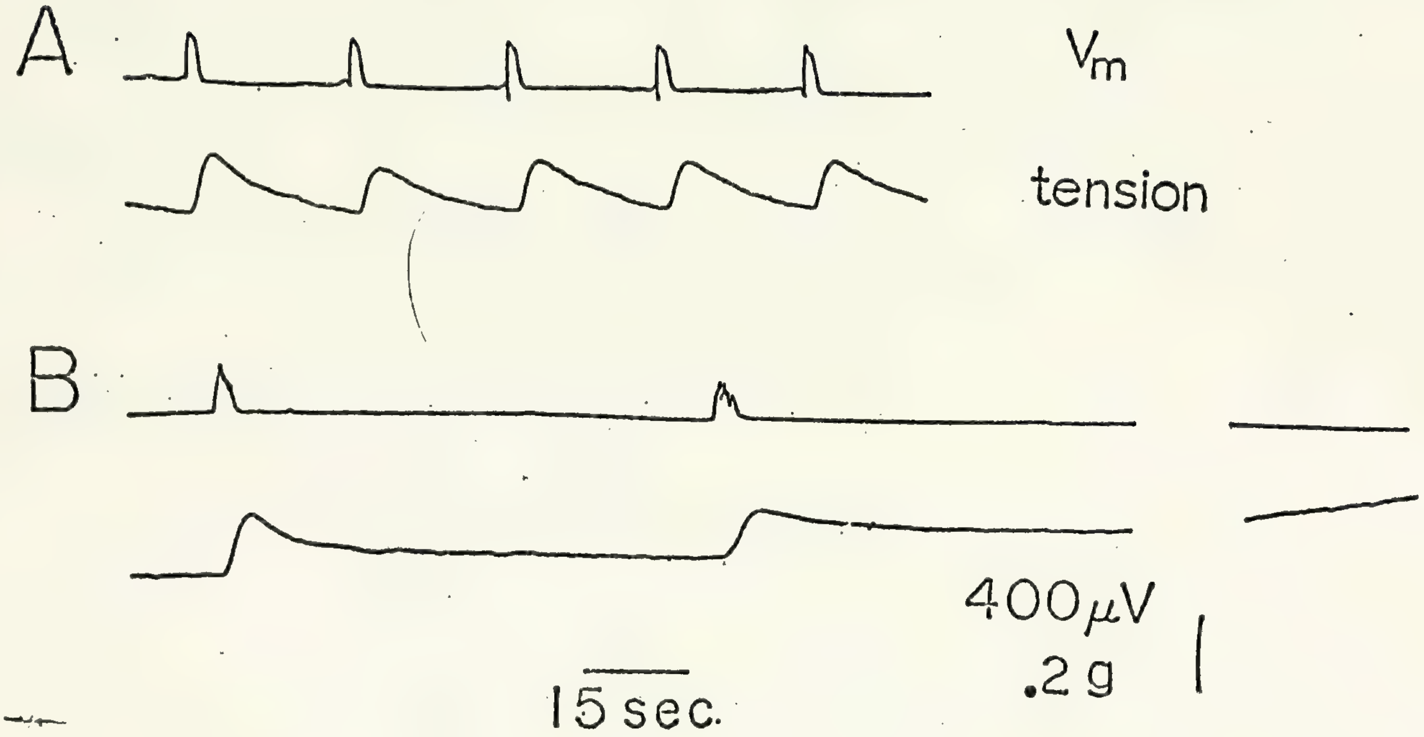

A
Vm
tension
B
400μV
.2 g
15 sec.

Figure 9. Application of electrical stimulation to a segment of intact longitudinal muscle remaining quiescent after 10 min incubation in high EGTA saline.

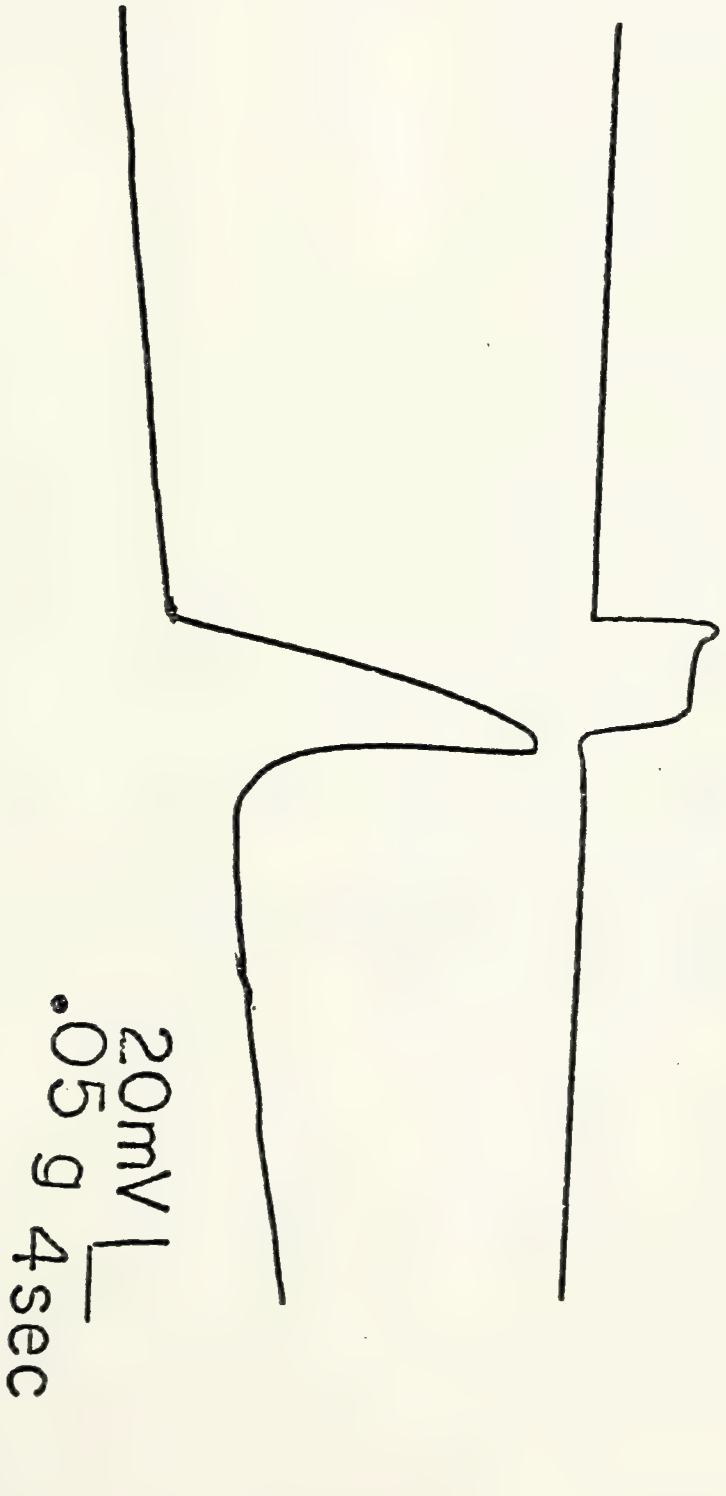

20mV
.05 g
4 sec

Figure 10. (A) Mechanical activity in control saline recorded from cat vena cava. (B) Resulting activity following incubation for 5 min in Ca^{2+} free saline. (C) Exposure for 23 min to Ca^{2+} free saline.

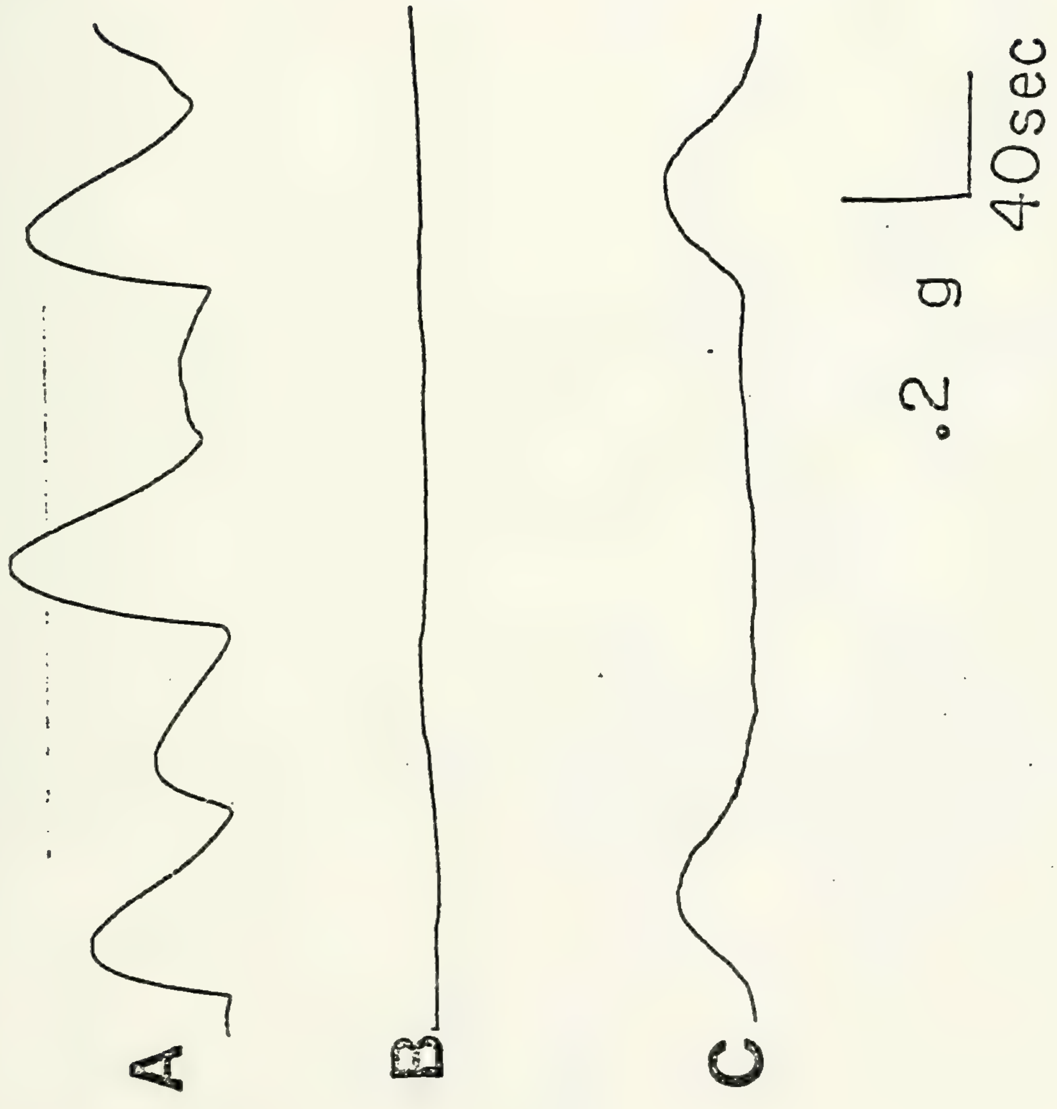

A
B
C
.2 g
40sec

Figure 11. (A) Mechanical activity in control saline recorded from cat stomach. (B) Resulting activity following incubation for 30 min in Ca^{2+} free saline. (C) Exposure for 35 min to Ca^{2+} free saline.

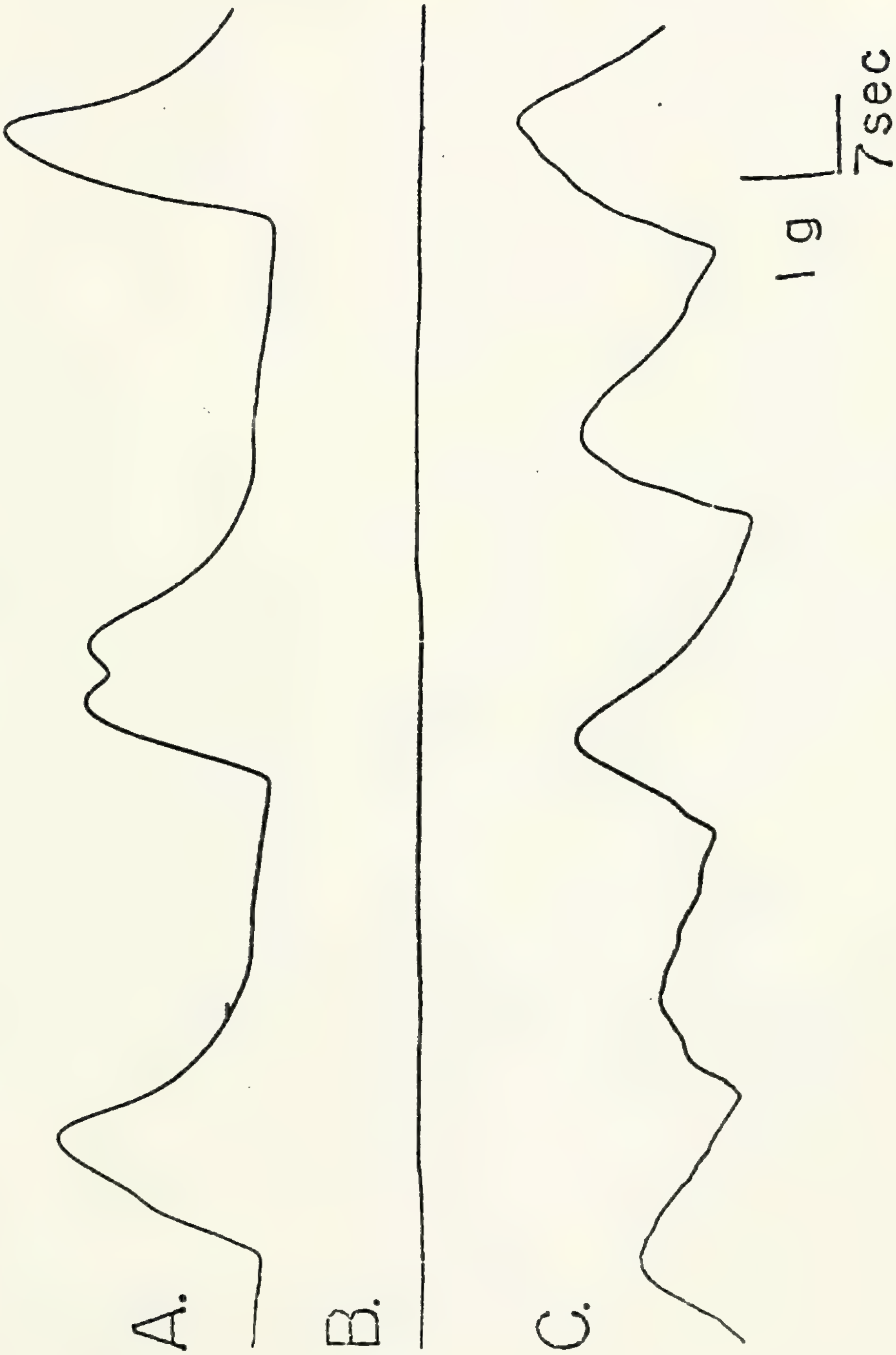

A.
B.
C.
1 g
7sec

Figure 12. (A) Mechanical activity in control saline recorded from cat colon. (B) Resulting activity following 20 min incubation in Ca^{2+} free saline. (C) Application of electrical stimulation to the preparation remaining quiescent after exposure for 25 min to Ca^{2+} free saline.

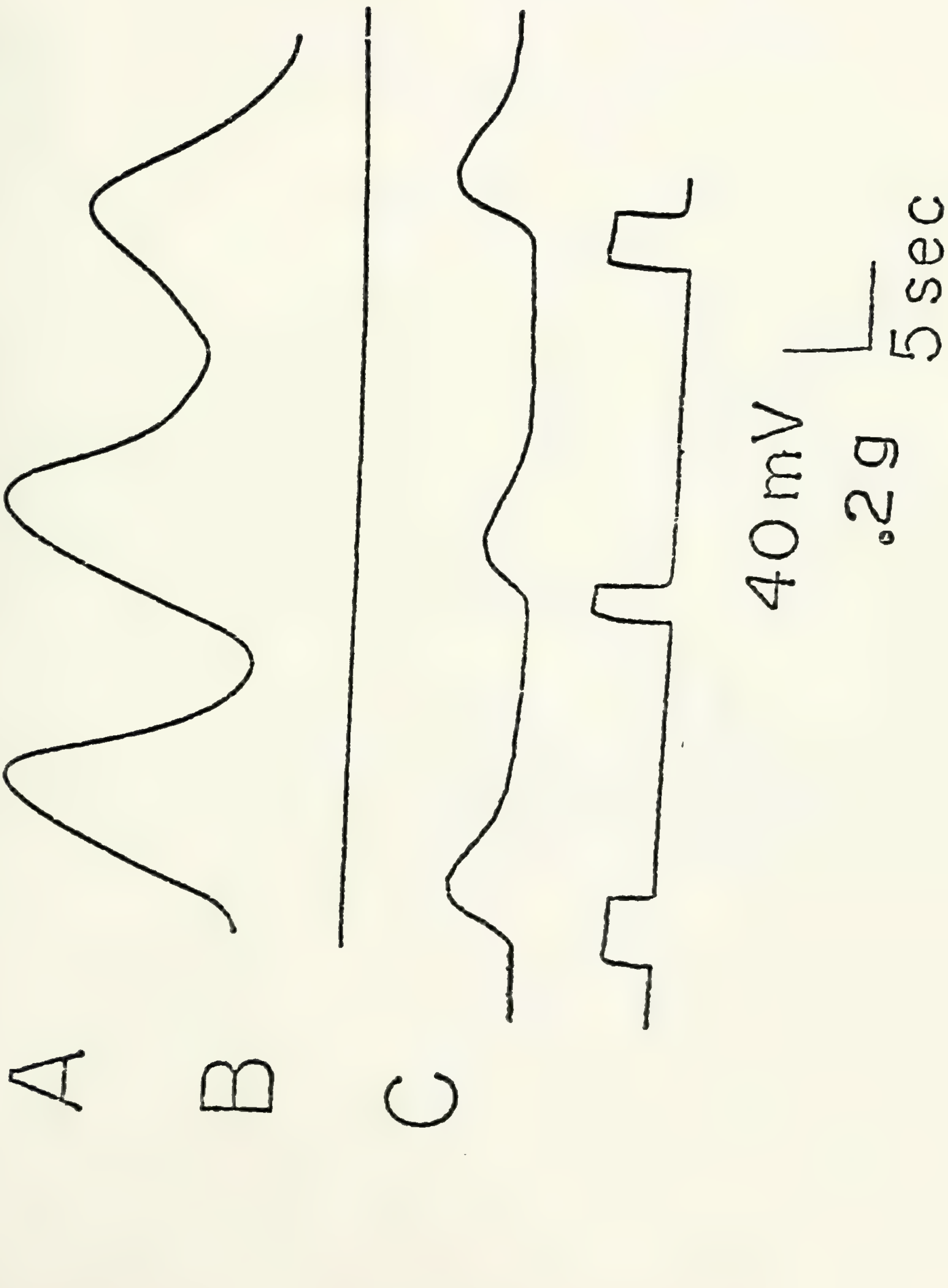
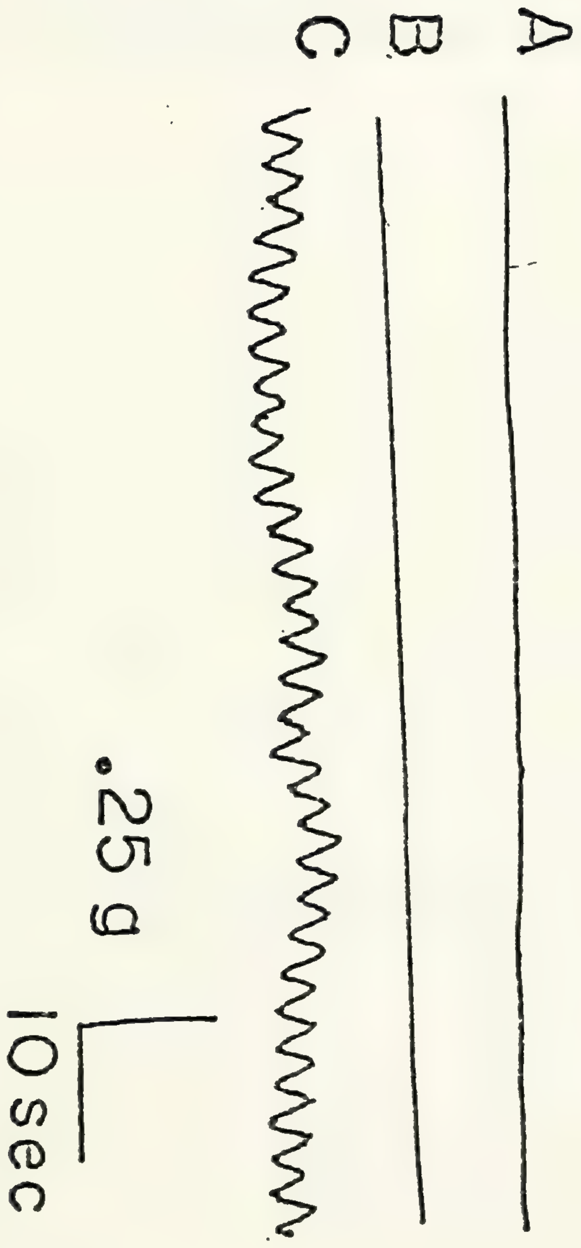

A
B
C
40mV
.2g
5sec

Figure 13. (A) Mechanical activity in control saline recorded from cat esophagus. (B) Resulting activity following 11 min incubation in Ca^{2+} free saline. (C) Resulting activity following addition of 2 mM BaCl after 16 min in Ca^{2+} free saline.


A
B
C
.25 g
10 sec

Rat small intestinal muscle does not develop prolonged potentials during incubation in Ca^{2+} free saline but, instead, fast rhythmic voltage changes (fast potentials) are produced (186). Accompanying fast potentials was a steady increase in resting tension rather than rhythmic contractile activity (n=4) (Figure 14). This tetanus-like state persisted for over 1 hour in some cases.

Figure 14. Pressure electrode recording of electrical (upper trace) and corresponding mechanical activity (lower trace) in a segment of rat small intestine following incubation in Ca^{2+} free saline for 30 min. (Cal. bar.: .2 mV, .05 gm, 3 sec)

B. Discussion

Rhythmic contractile activity was found to persist in several smooth muscle preparations in the absence of extracellular Ca^{2+}. I am confident that extracellular Ca^{2+} was removed for the following reasons: (a) exposure of smooth muscle preparations to 3-5 mM EGTA has been shown repeatedly to remove Ca^{2+} from the extracellular space and extracellularly bound Ca^{2+} (84,85,292). (b) From application of high EGTA levels the possible presence of a "protected" extracellular Ca^{2+} store such as in the caveolae was eliminated (17).

The trigger for the contractile activity in Ca^{2+} free saline is depolarization of the plasma membrane. Depolarization of the plasma membrane either spontaneously by prolonged potentials or induced by electrical stimulation, BaCl, ACh, or K^+ was equally effective in generating contractions. Thus, depolarization of the membrane may produce contractions in smooth muscles incubated in Ca^{2+} free saline by releasing intracellularly stored Ca^{2+}. Possible candidates for intracellular storage sites of Ca^{2+} in smooth muscle are: sarcoplasmic reticulum, mitochondria, and endoplasmic reticulum (94,117,141,152,227,240,274,291). Since the bulk of the contractile proteins are at a distance "far" from the plasma membrane, this internal Ca^{2+} store may be partitioned away from the plasma membrane. However, a functional link between membrane potential changes and the release of the stored Ca^{2+} is indicated.

The spontaneous mechanical response to EGTA treatment differs in rat small intestine from the other preparations tested. I attribute this difference to the different nature of the spontaneous electrical activity generated by rat and cat small intestine during incubation in Ca^{2+} free saline

rather than an inherent difference in the nature of the Ca^{2+} stores. The normally slow contracting rat small intestine may not be able to contract at a rate corresponding to fast potentials. Hence, a state similar to tetanus results from the fast rhythmic membrane depolarizations.

The possibility of intracellular sources of Ca contributing to the activation of contraction in smooth muscle has been postulated elsewhere. Bozler (36) found that contractions in rabbit aorta were induced by K^+ depolarization even after preparations were incubated in Ca^{2+} free saline for 3 hours. Somlyo et al. (275) observed spontaneous contractions of turtle oviduct that persisted for approximately 30 min in Ca^{2+} free saline. Golenhofen (119) found tonic contractions, triggered by ACh, to persist in dog stomach during incubation in Ca^{2+} free saline. In contrast, others have reported that rhythmic mechanical activity was eliminated by EGTA treatment (5,59,94,95,142). Most of these observations had been made during a time corresponding to the transition period between normal and EGTA-induced activity. Recordings made at later times might have demonstrated rhythmic contractile activity.

In the BA experiments the possibility exists of direct activation of the contractile apparatus by Ba^{2+}. I have discounted this possibility based on the relative concentrations of ions involved. Ebashi et al. (cited in 187) showed that myosin B preparations from chicken gizzard and bovine aortic muscles are approximately 200 times less sensitive to Ba^{2+} than to Ca^{2+}. Ba-induced contractions in Ca^{2+} free saline can be as large as control contractions, suggesting that internal Ba^{2+} levels would have to approximate 1 mM during peak contractions. It is much more likely that Ba^{2+} acts by supporting large action potentials (301) and that the plasma membrane depolarization

results in a release of internal Ca^{2+}. The action of Ba^{2+} would then be similar to direct electrical stimulation or prolonged potentials.

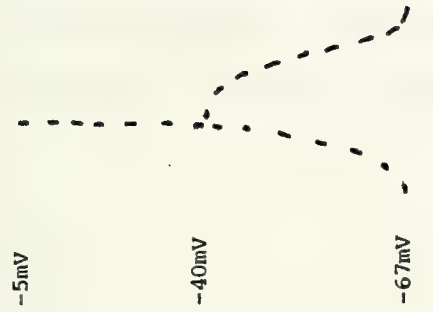
Under physiological conditions, contractions in smooth muscle are triggered by spikes. Intracellular recordings from cat small intestinal muscle show spikes near the peak of slow waves. For intact cat longitudinal muscle the mean resting potential is approximately -67 mV; mean slow wave amplitude 27 mV; and maximum spike amplitude 35 mV (80) (see Figure 15). The membrane potential profile for prolonged potentials is similar to that of spikes (Figure 15). In Ca^{2+} free saline the resting membrane potential of cat small intestine depolarizes to around -36 mV (234). The peak potential attained by large amplitude prolonged potentials is approximately 0 mV (234). Thus, the voltage excursion of both prolonged potentials and spikes is approximately 35-36 mV over the same range of membrane potential.

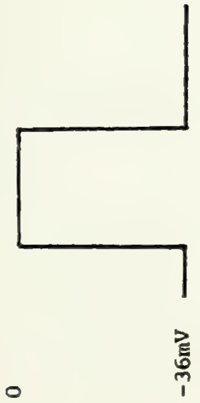
Since the average amplitude of contractions in Ca^{2+} free saline constitutes a significant fraction of control contraction amplitudes, a major source of the Ca^{2+} involved in activation of contraction in smooth muscle may be released from voltage sensitive intracellular pools. From the near identical magnitudes of spikes and prolonged potentials over the same range of absolute membrane potential, voltage dependent release of intracellular Ca^{2+} could occur under physiological conditions. Hence release of intracellular Ca^{2+}, following depolarization of the plasma membrane, may represent a fundamental step in the excitation-contraction coupling mechanism in smooth muscle.

Recent morphological studies (117,274) show an abundance of sarcoplasmic reticulum near the plasma membrane of smooth muscle cells. As in striated muscle, the excitation of the plasma membrane may release stored Ca from

intracellular depots within smooth muscle cells. Analysis of total tissue Ca in smooth muscle shows a value of .1-.5 mM, approximately 100 to 1000 times larger than the level of ionized Ca needed for activation of contraction (58,153). Although a significant portion of this total Ca is probably membrane bound, the remainder may constitute an intracellular store of sufficient magnitude to support contractile activity.

Figure 15. Membrane potential profiles of prolonged potentials and spikes. Note that prolonged potentials and spikes are approximately the same amplitude and occur over approximately the same range of absolute membrane potential.


-5mV
-40mV
-67mV


0
-36mV

C. Metabolic Correlates

Summary

(1) A population of mitochondria is present within 200Å of the inner surface of the plasma membrane in longitudinal muscle cells.

(2) Slow wave production requires oxidative phosphorylation remain intact, while contractions can utilize either oxidative or glycolytic energy sources.

C. Metabolic Correlates

(i) Metabolic Inhibitors

Spontaneous electrical and mechanical activity in control saline is illustrated in Figure 1. Contractions usually accompanied spikes triggered during the slow wave peak. Addition of 1 mM $BaCl_2$ (n=18) to mechanically quiescent preparations, resulted in production of large spikes during the slow wave depolarization and accompanying contractions (Figure 2).

Addition of 1 mM NaCN (n=5) to saline containing BaCl resulted in elimination of slow wave activity within one minute, but Ba-induced spikes and contractions persisted for up to thirty minutes (Figure 3). Eventually all electrical and mechanical activity ceased. The persistence of Ba-induced contractions during CN^- treatment was also present in isolated longitudinal muscle (Figure 4).

Following a 5 min incubation period in saline containing 1 mM BaCl and 1 mM NaCN, .25 g/l methylene blue was added (n=7). In 3 of the 7 preparations, after methylene blue addition electrical and mechanical activity progressively increased in frequency until, after 24 min in this solution, the frequency of electrical and mechanical events were similar to control frequency (e.g., see Figure 5). These membrane potential oscillations were ouabain sensitive (2 of 2 preparations tested), as are normal slow waves but not Ba-induced spikes (n=6). In the remaining 4 preparations, activity did not increase in frequency following methylene blue but eventually ceased within 30 min of the initial exposure to NaCN. Two preparations were tested in which solutions did not contain BaCl (i.e., saline contained 1 mM NaCN and .25 g/l methylene blue).

Figure 1. Spontaneous electrical (V_m) and mechanical activity in a segment of intact small intestine. Electrical activity is recorded with a tube electrode. Intestine is in its normal configuration, i.e., longitudinal muscle faces outward, circular muscle faces the intestinal lumen.

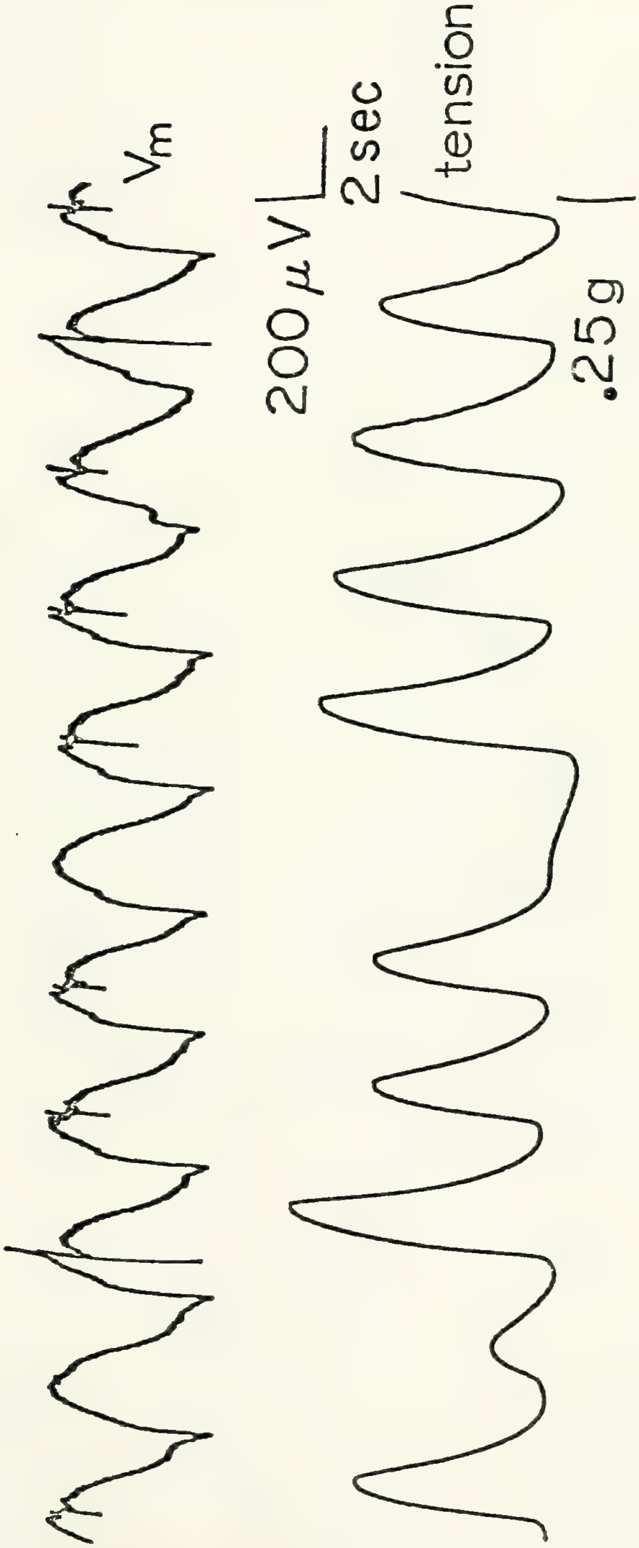

Vm
200 μV
2 sec
tension
.25g

Figure 2. Spontaneous electrical and corresponding mechanical activity in a segment of intact intestine. Electrical activity is recorded with a tube electrode. Intestine is in its normal configuration. (A) Activity in control saline. (B) Following 1 mM Ba^{2+} addition.

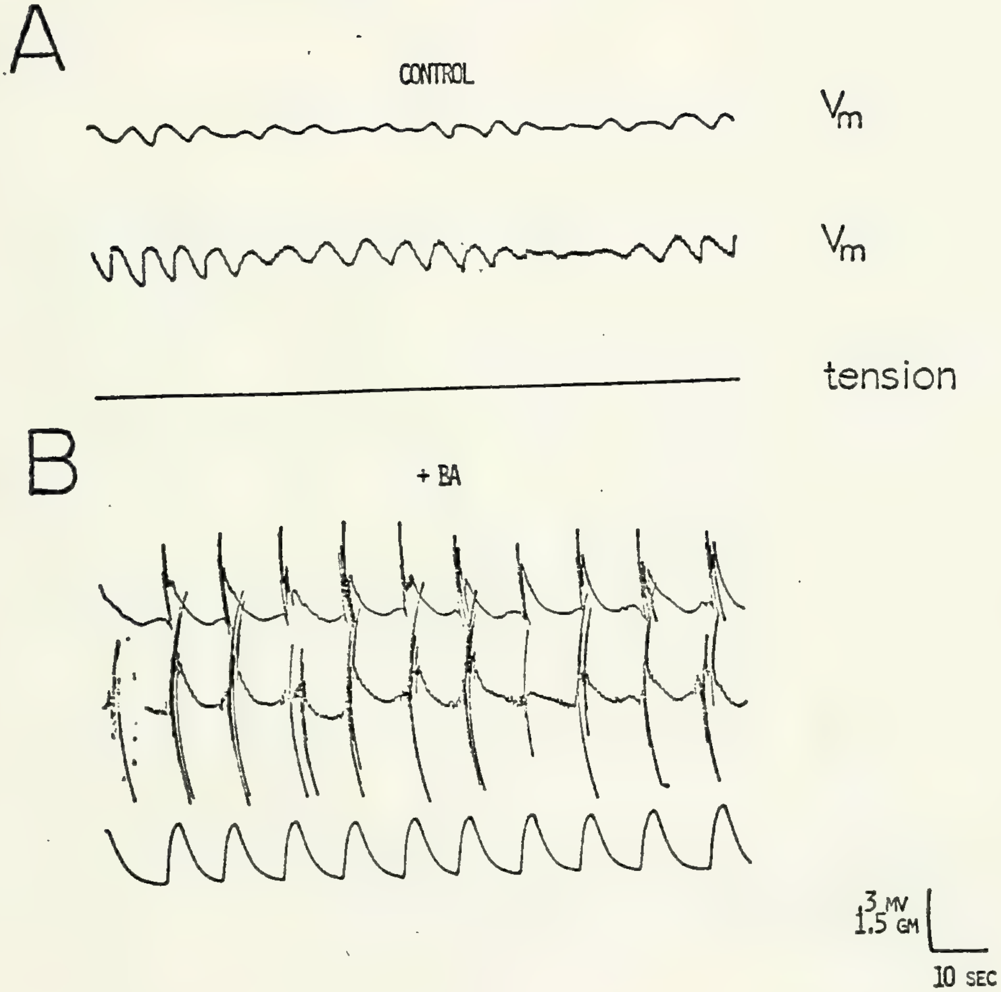

A
CONTROL
Vm
Vm
tension
B
+ BA
3 MV
1.5 GM
10 SEC

Figure 3. (A) Control electrical and mechanical activity in a segment of intact intestine. Electrical activity is recorded with a tube electrode. Intestine is in its normal configuration. (B) Activity resulting from 4 min exposure to 1 mM BaCl. (C) Activity resulting from 1 min incubation in saline containing 1 mM BaCl and 1 mM NaCN. (D) 25 min incubation in saline with Ba^{2+} and CN^-.

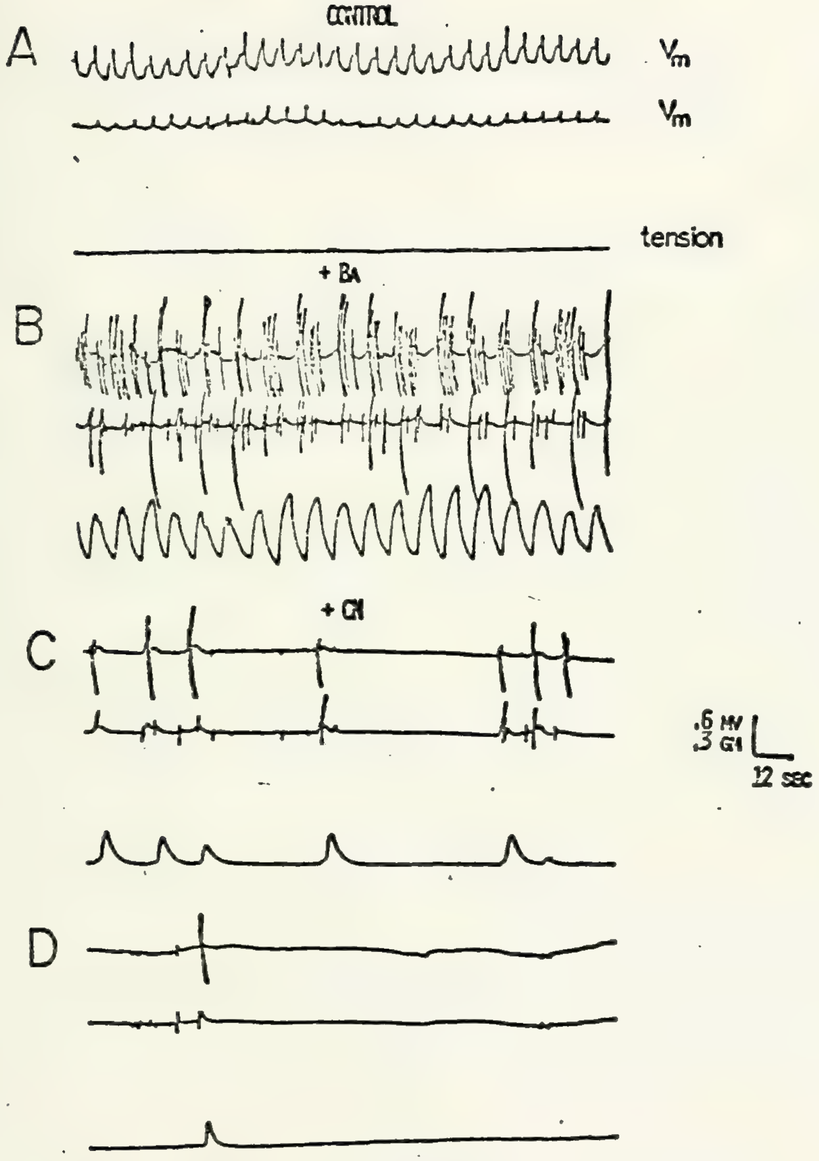

CONTROL
A
Vm
Vm
tension
+ BA
B
+ CN
C
.6 MV
.3 GM
12 sec
D

Figure 4. Electrical (A) and mechanical (B) activity recorded in isolated longitudinal muscle following exposure to 1 mM BaCl and 1 mM NaCN for 10 min. Electrical activity is recorded with a tube electrode.

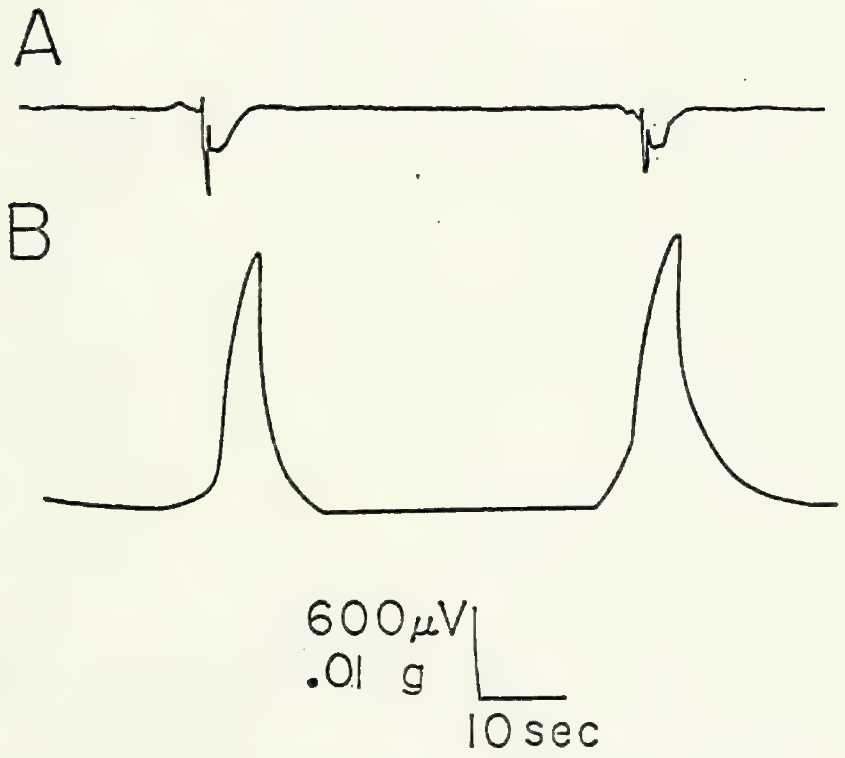
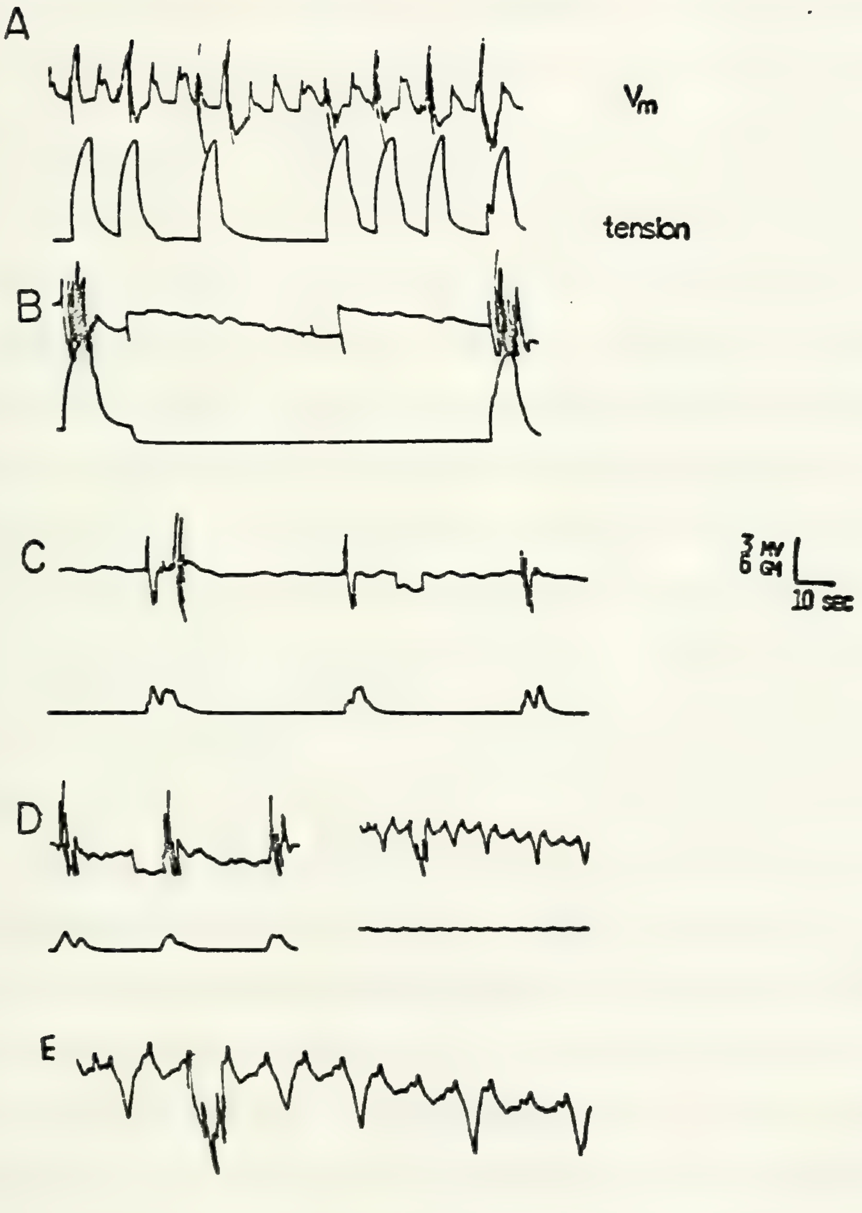

A
B
600µV
.01 g
10 sec

Figure 5. (A) Electrical (V_m) and corresponding mechanical activity in saline containing 1 mM BaCl. Electrical activity is recorded with a tube electrode. The intact intestine is in its normal configuration. (B) Activity resulting from 3 min exposure to saline containing 1 mM BaCl and 1 mM NaCN. (C) The activity which results from addition of .25 g/l methylene blue to the stock saline containing 1 mM BaCl and 1 mM NaCN. This preparation has been exposed to 1 mM BaCl and 1 mM NaCN for 5 min and 1 mM BaCl, 1 mM NaCN and .25 g/l methylene blue for 6 min. (D) (left) 10 min in methylene blue; (right) 25 min in methylene blue. (E) An enlargement of trace D (right).


A
Vm
tension
B
C
3 mV
6 GM
10 sec
D
E

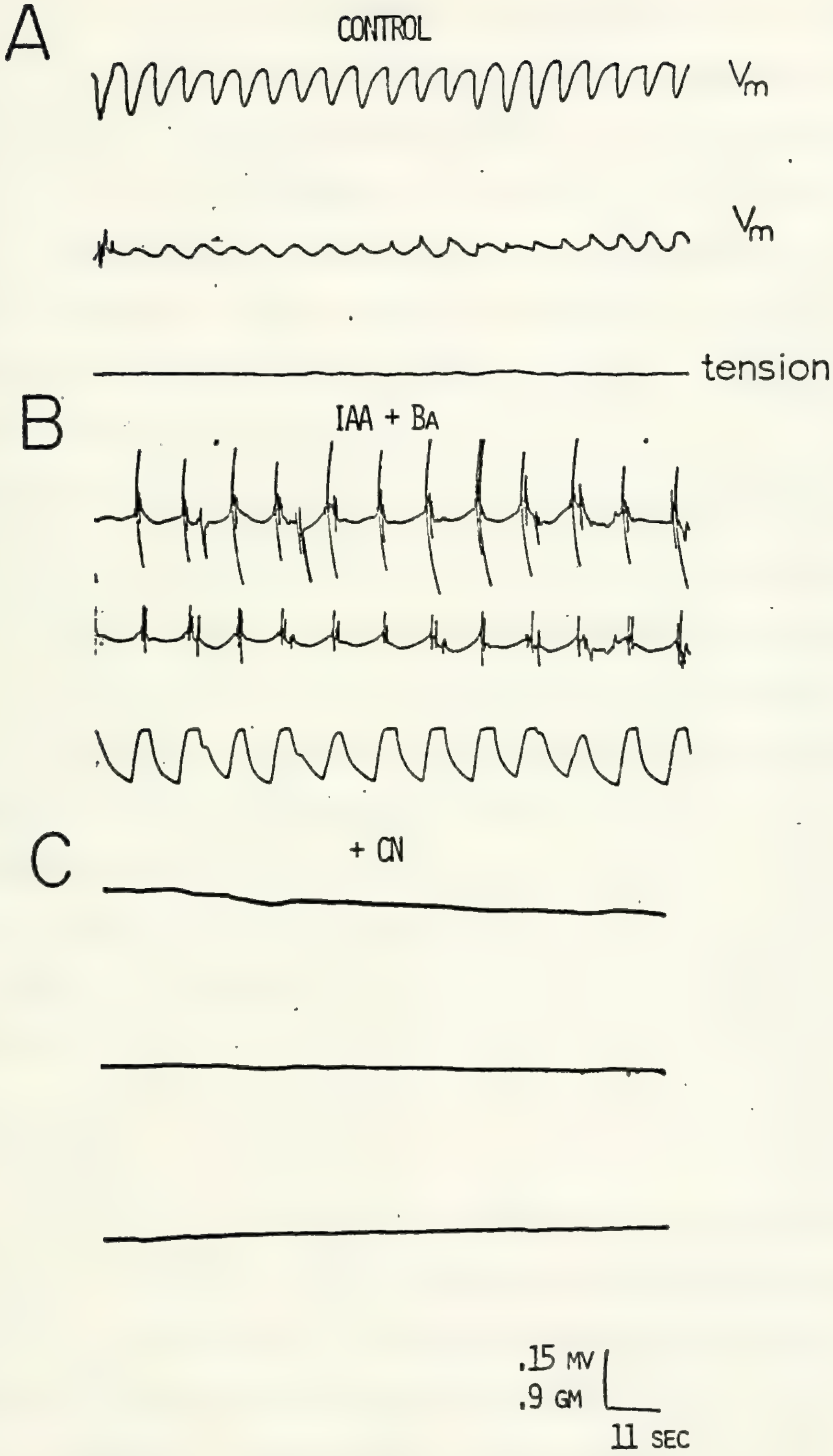
In both cases slow rhythmic activity was not restored by methylene blue.

Methylene blue acts as an alternate elctron acceptor in the electron transport chain and may function as a bypass to the block in the chain caused by CN^- (73). If CN^- eliminated slow waves by disruption of ATP production, then reversal of the CN^- block should result in resumption of slow wave activity. The activity resulting from treatment with methylene blue had a period similar to slow waves and possessed the same sensitivity to ouabain.

Contractions and slow waves persisted for hours in the presence of iodoacetic acid (IAA). Following cyanide addition to saline containing IAA plus BaCl, activity was eliminated within 45 sec, in 4 of 5 preparations (see Figure 6). If the reverse sequence was followed, i.e., IAA addition to preparations containing NaCN plus BaCl, identical effects were observed. In 1 of 5 preparations Ba spikes persisted for 2 minutes in the presence of NaCN plus IAA, but there was no accompanying mechanical activity.

From the above results it can be concluded that for slow wave generation to continue, oxidative phosphorylation must remain functionally intact. This dependency might take on 2 forms. First, slow waves may directly use oxidatively produced ATP in their genesis. Thus, inhibition of oxidative phosphorylation would directly disrupt slow wave production by elimination of the source of ATP. Second, either oxidative or glycolytic sources of ATP may be used directly for generation of slow waves, but a basal level of ATP must be present for slow waves to be produced. Inhibition of oxidative phosphorylation may bring the level of ATP below this basal level and slow waves would, therefore, not be produced. At present I can not distinguish between these possibilities. Contractions can occur in the absence of either glycolysis or oxidative phosphorylation.

Figure 6. (A) Electrical (V_m) and corresponding mechanical activity in control saline. Electrical activity is recorded with a tube electrode. The intestine is in its normal configuration. (B) Activity resulting from exposure to saline containing 10 mM IAA and 1 mM BaCl. (C) Activity resulting from 35 sec exposure to 1 mM NaCN, 10 mM IAA and 1 mM BaCl.


A
CONTROL
Vm
Vm
tension
B
IAA + BA
C
+ CN
.15 MV
.9 GM
11 SEC

There is a possible complication with investigating the effects of metabolic inhibitors on Ca sensitive events, such as slow waves, in that the addition of metabolic inhibitors has been shown to cause the release of internally stored Ca (13,25). The elimination of slow waves by CN^- probably does not result from an increase in internal Ca^{2+} levels, but rather from interruption of ATP synthesis. This conclusion is based on the different effects which metabolic inhibitors and high Ca_i^{2+} levels have on slow waves. Increasing Ca_i^{2+} produced changes in slow wave frequency with the slow waves characterized by a prolonged systolic and shortened diastolic phase compared to control activity. In contrast, slow wave frequency was usually not affected by metabolic inhibitors but slow wave amplitude was reduced. The effects of hypoxia and 10^{-5} M DNP on slow waves are illustrated in Figures 7 and 8. In both cases slow wave amplitude was markedly affected but frequency remained relatively unchanged. Usually, following NaCN addition, slow wave amplitude not frequency was affected. In a few cases, slow wave frequency was reduced by CN^-. In these preparations the diastolic not systolic, phase of the slow wave was more prolonged than under control conditions (Figure 9).

(ii) NADH Studies

In a previous study, oscillations in NADH levels were correlated with slow waves, i.e., an oscillation in NADH levels had a period similar to slow waves (78). Fluorescence signals gathered from longitudinal muscle of intact segments, with simultaneous electrical recordings, are illustrated in Figure 10. Typically, oscillations in NADH fluorescence levels could be observed with fewer than 30 averages. When the intestine was everted, and the fluorescence signal gathered from circular muscle, no oscillation in

Figure 7. (A) Pressure electrode recording of slow waves from intact longitudinal muscle. Saline has 20 ppm oxygen. (B) Activity resulting from exposure to saline with 10 ppm oxygen. (C) Saline with 4 ppm oxygen. (D) Return to control saline, 20 ppm oxygen.

Effect of Hypoxia

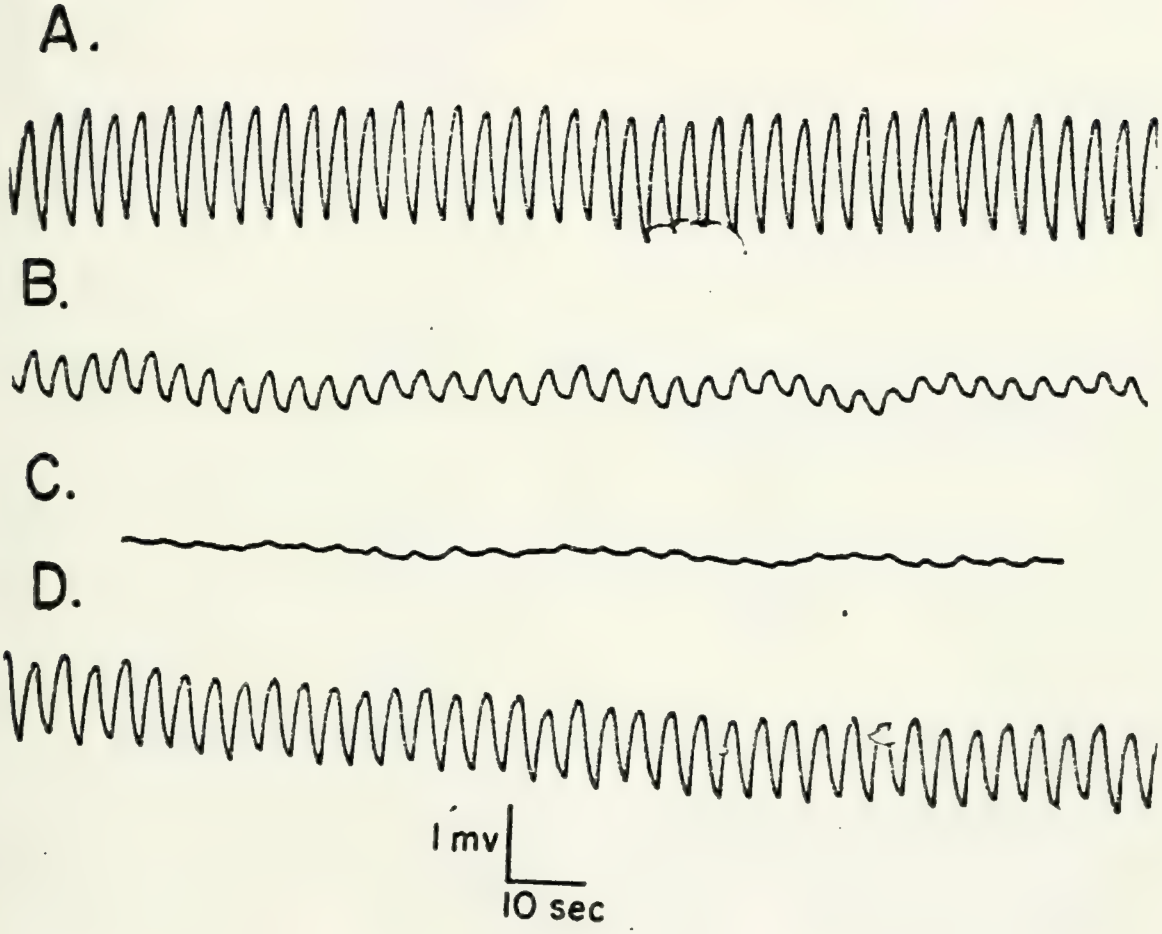


Figure 8. Microelectrode recording from intact longitudinal muscle. At the beginning of the trace (arrow) saline containing 10^{-5} M DNP was started to flow.

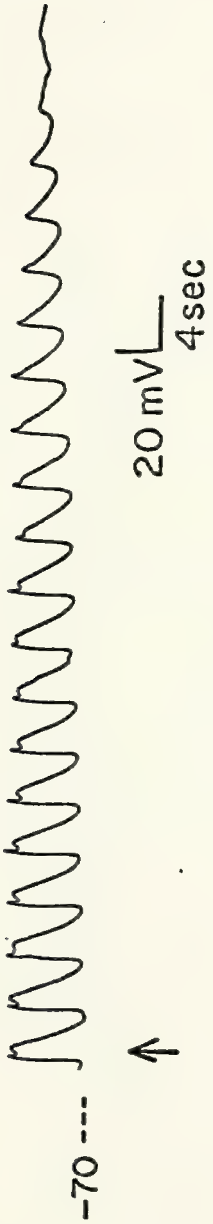

-70
20 mV
4 sec

Figure 9. (A) Pressure electrode recording of slow waves from intact longitudinal muscle. (B) Activity resulting from 5 min exposure to .2 mM NaCN.

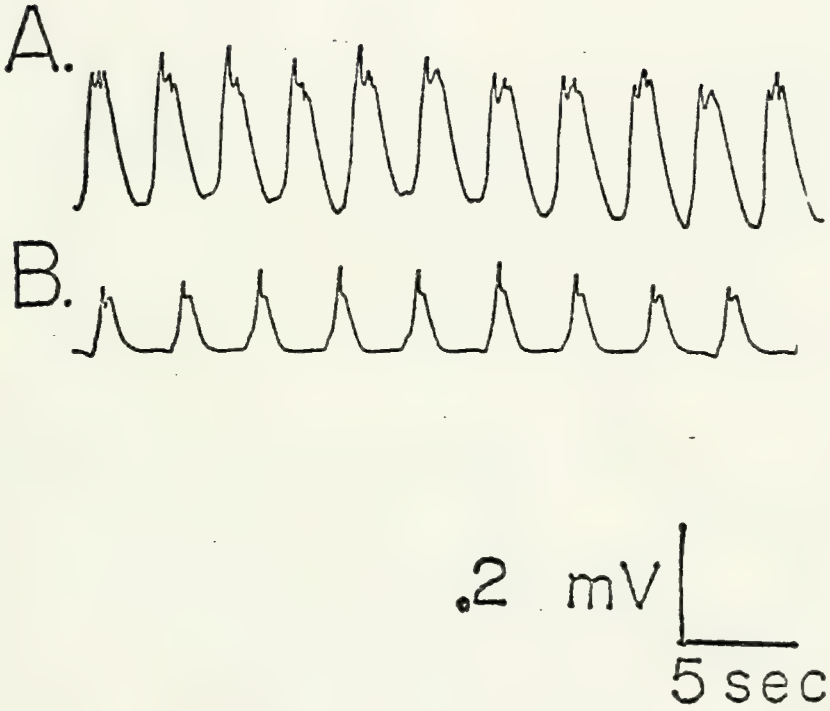
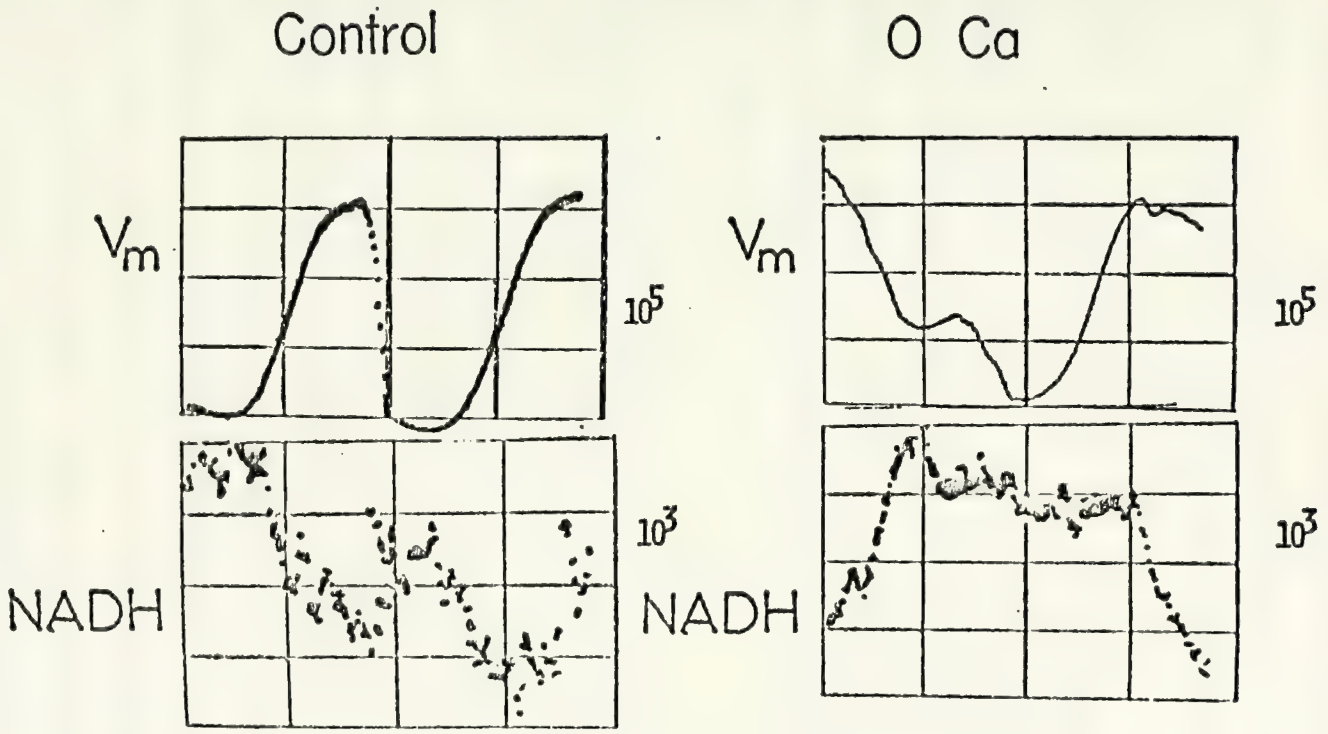

A.
B.
.2 mV
5 sec

Figure 10. Summed voltage (V_m, upper) and fluorescence (NADH, lower) signals from intact muscle using a computer of average transients. For these records electrical recordings were with a tube electrode and the intestine was in its normal configuration. The control record is an average of 33 slow waves; the record from 0 Ca^{2+} saline is an average of 30 slow waves. Gain is given in arbitrary units for each record; one horizontal division corresponds to 2 sec.


Control
O Ca
V_m
10^5
NADH
10^3
V_m
10^5
NADH
10^3

fluorescence could be resolved (Figure 11); although I routinely could record rhythmic electrical and mechanical activity in the everted segments.

In an effort to examine possible coupling between slow waves and NADH oscillations, the effects of treatments which alter slow wave frequency were examined with respect to the NADH fluorescence oscillations. Illustrated in Figure 10 are voltage and fluorescence signals before and after exposure to 0 Ca^{2+} saline (n=6). In 0 Ca^{2+} saline the frequency of slow waves and the NADH fluorescence oscillations were slower than in normal saline. Identical effects were observed following addition of 1 mM Mn^{2+} to normal saline (n=3). Addition of 2 mM d-cAMP (n=5) to saline made hypertonic with sucrose, accelerated the frequency of both voltage and fluorescence oscillations (Figure 12).

(iii) Electron Microscopy Studies

Mitochondria in smooth muscle have been shown to be localized into 2 general regions: (1) near the ends of the nucleus and (2) close to the inner surface of the plasma membrane (274,275,282). Electron micrographs of cat intestine were examined for the purpose of studying mitochondrial distribution close to the plasma membrane. The results are summarized in Table 1. In the longitudinal muscle layer a population of mitochondria was present within 200Å of the inner surface of the plasma membrane. This population was preferentially found in cells of the longitudinal muscle layer over the circular muscle layer and no significant difference existed between the 2 layers at distances greater than 600Å.

(iv) Agents Which Alter Contractile Activity

Contractions have been eliminated from spontaneously active preparations

Figure 11. Summed voltage (V_m, upper) and fluorescence (NADH, lower) signals from intact muscle. For this record electrical recordings were with a tube electrode and the intestine was in the everted configuration (devoid of muscularis mucosa). This record is an average of 31 slow waves. One horizontal division corresponds to 2 sec.

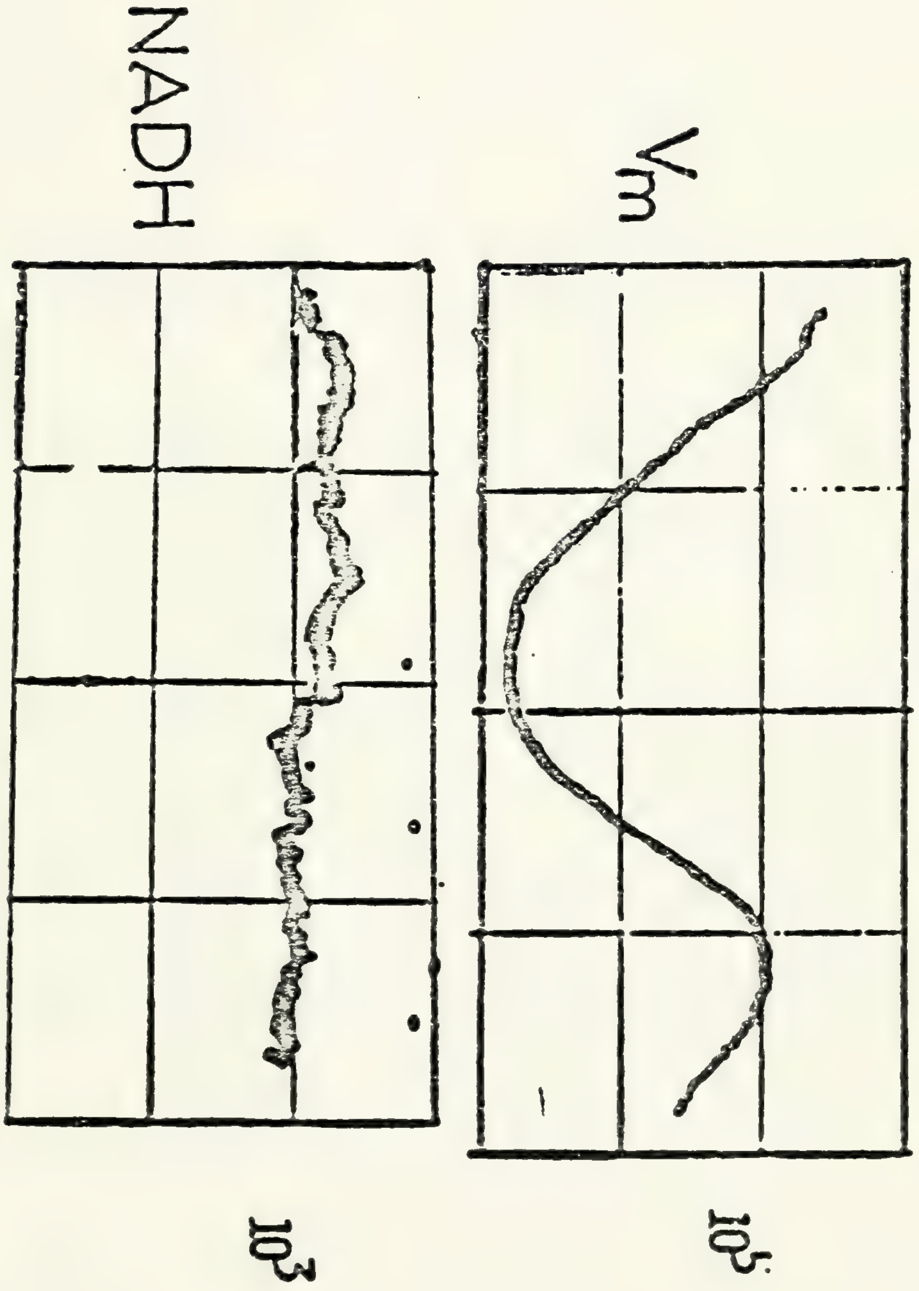

NADH
Vm
10³
10⁵

Figure 12. Summed voltage (V_m, upper) and fluorescence (NADH, lower) signals from intact muscle. For these records electrical recordings were with a tube electrode and the intestine was in its normal configuration. The control record is an average of 40 slow waves; the d-cAMP record an average of 27 slow waves. In this experiment both control and experimental solutions contained 50 g/l sucrose. 2 mM d-cAMP was added to the experimental solution. One horizontal division corresponds to 2 sec.

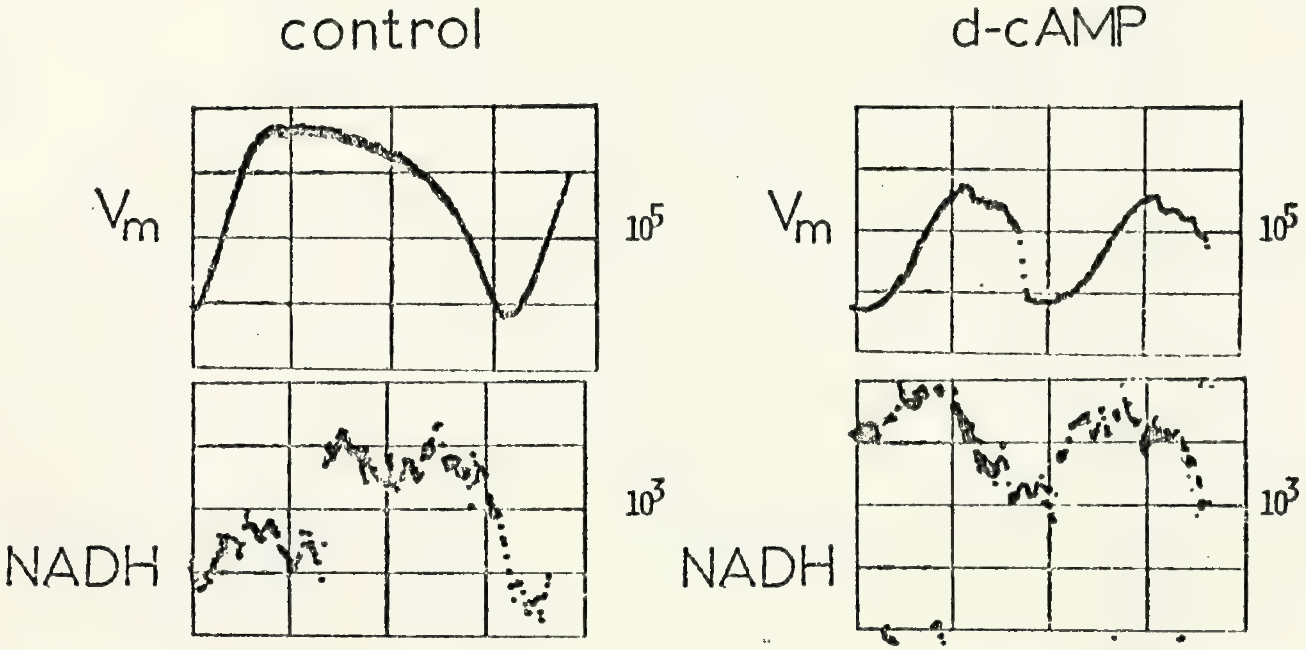

control
d-cAMP
V_m
10^5
NADH
10^3
V_m
10^5
NADH
10^3

Table 1. Mitochondrial Distribution.

Peripheral mitochondria or mitochondria in the peripheral ring corresponds to mitochondria within 200Å of the inner membrane surface.

	LONGITUDINAL MUSCLE	CIRCULAR MUSCLE
#CELLS	161	111
CELLS WITH ONE MITOCHONDRION IN THE PERIPHERAL RING	64	8
CELLS WITH MORE THAN ONE PERIPHERAL MITOCHONDRION	18	3

Figure 13. (A) Control electrical (V_m) and mechanical activity. Electrical activity was recorded from intact intestinal muscle, in the normal configuration with a tube electrode. (B) Activity resulting from 7 min exposure to 3 mM caffeine.

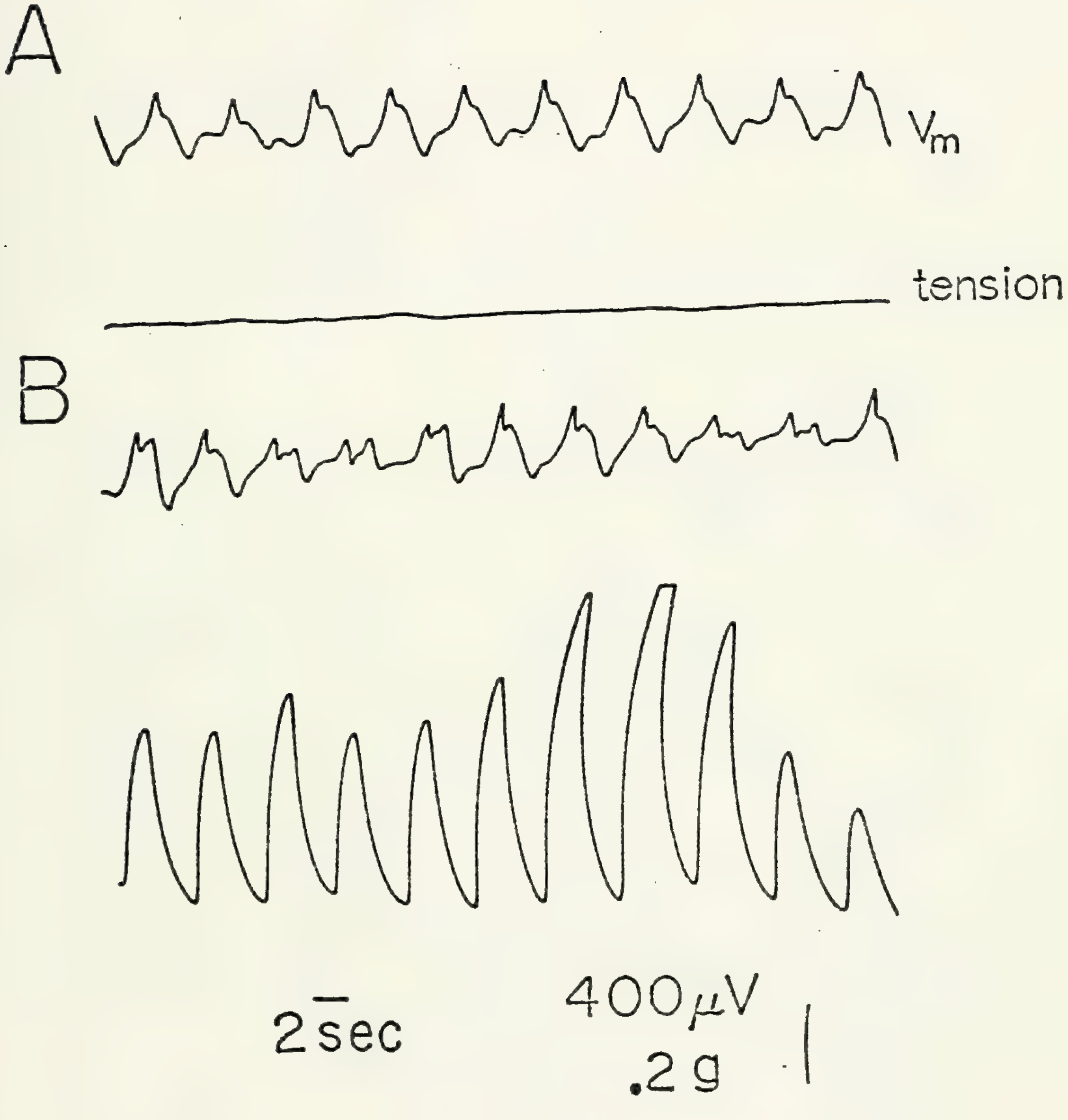
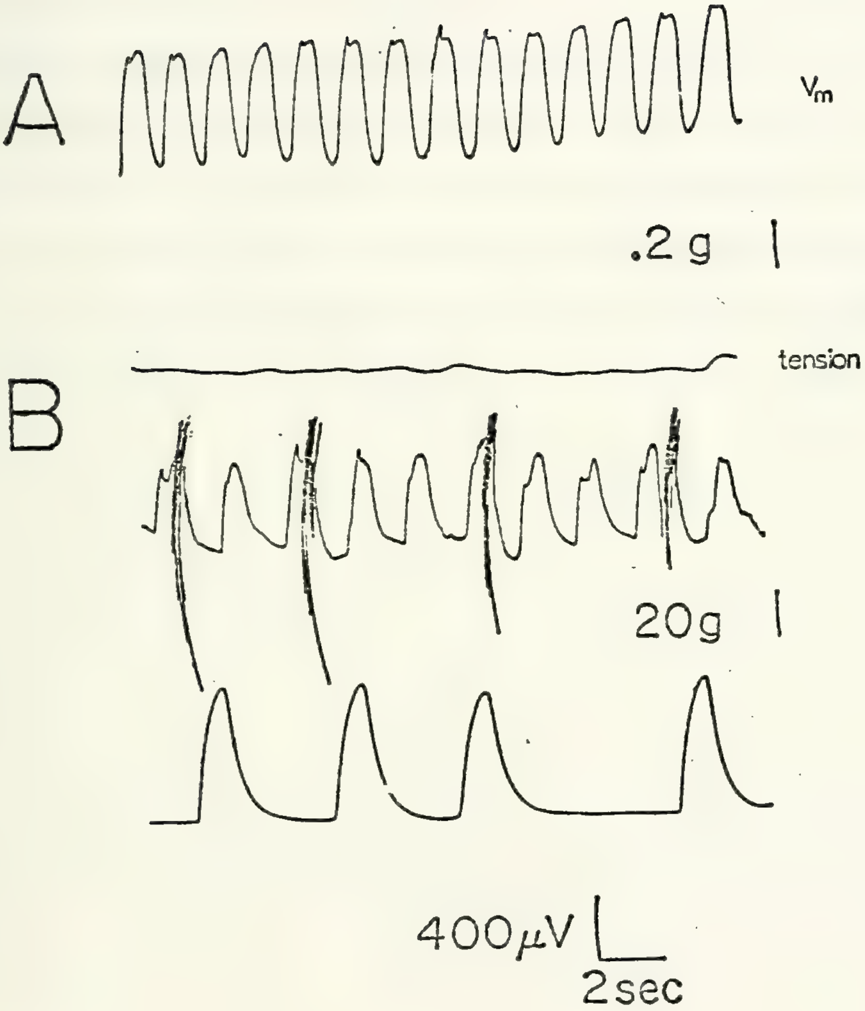

A
Vm
tension
B
2 sec
400 μV
.2 g

Figure 14. (A) Control electrical (V_m) and mechanical activity. Electrical activity was recorded from intact intestinal muscle in the normal configuration with a tube electrode. (B) Activity resulting from 1 min exposure to 10^{-4} M eserine.


A
Vm
.2 g
tension
B
20g
400μV
2sec

by addition of atropine (184) or induced in quiescent preparations by caffeine (290). Application of 10^{-5} M atropine (n=10) or 3 mM caffeine (n=4) (Figure 13) drastically altered contractile activity. In either case (caffeine, atropine) slow wave frequency was not changed. Application of agents which, in addition to inducing large changes in contractile activity, altered membrane spiking, produced changes in slow wave frequency. Following $BaCl_2$ (see Figure 2) or eserine (Figure 14) (n=5) increases in the level of membrane spiking and decreases in slow wave frequency occurred. When changes in the degree of membrane spiking did not accompany these treatments (1 of 5 eserine treated preparations; 2 of 18 Ba^{2+} treated preparations) slow wave frequency was not changed.

C. Discussion

Following CN^- application, slow wave activity is eliminated within 1 min but evoked contractions persist for up to 30 min. CN^- addition in the presence of IAA results in the immediate termination of all contractile activity, while in the presence of IAA alone contractions persist for hours. These results suggest that following inhibition of oxidative phosphorylation contractions can be supported for a substantial time period by glycolysis alone, while slow waves cannot. Energy produced exclusively by glycolysis is sufficient to support contractions in other preparations also (48,179,226,229, 270). That the mechanisms responsible for setting slow wave frequency and for generation of contractions do not utilize the same energy pool, or have substantially different sensitivities to alterations in the level of ATP in a common pool, is supported by the studies in which contractile activity was markedly changed and the effects on slow wave frequency observed. Following atropine contractile activity is totally eliminated, while after caffeine addition contractions are markedly enhanced. Following either treatment slow wave frequency is not changed. If the mechanisms responsible for determination of slow wave frequency and generation of contractions utilize a common energy pool with equal sensitivities to changes in the energy pool, then treatments which cause marked changes in contractile activity (i.e., marked changes in ATP utilization) should alter slow wave frequency.

Since oxidative phosphorylation is involved in slow wave generation, an appealing possibility is that the population of mitochondria located within 200Å of the plasma membrane may be involved in slow wave generation.

These mitochondria are preferentially found in the longitudinal muscle layer, the site of slow wave generation, and by virtue of their close proximity to the plasma membrane, they may result in the formation of a compartmental region.

IV. SUMMARY AND CONCLUSION

A. Summary

Evidence has been presented for: (1) attributing a role to intracellular Ca^{2+} in the generation of intestinal slow waves. (2) Intracellular partitioning of Ca^{2+} and (3) a possible link between mitochondrial distribution and function and the generation of slow waves. The evidence is summarized below:

(1) Treatments which alter intracellular Ca^{2+} produce changes in the frequency and waveform of slow waves. Qualitatively identical results are observed following: reductions in Ca_o^{2+}, addition of Ca channel blockers, increases in Mg_o^{2+} or increases in internal pH, all treatment which decrease Ca_i^{2+}. Likewise, various treatments which increase Ca_i^{2+}: increasing Ca_o^{2+}, d-cAMP addition, or decreasing internal pH, all have similar effects on slow waves.

(2) Reductions in Ca_i^{2+} produce a decrease in slow wave frequency, with slow waves characterized by prolonged diastolic phases.

(3) Elevations in Ca_i^{2+} initially produce an increase in slow wave frequency, while further increases in Ca_i^{2+} produce a decrease in frequency. In both cases, the systolic phase of the slow wave is prolonged and the diastolic phase shortened compared with control activity.

(4) Increases in Ca_i^{2+}, via an increased transmembrane influx of Ca^{2+} (e.g., by BaCl or eserine), decrease slow wave frequency. However, increases in Ca_i^{2+}, by release of stored Ca^{2+} (e.g., caffeine), does not alter slow wave frequency.

(5) CTC fluorescence studies indicate an oscillation in Ca_i^{2+}, probably in the immediate vicinity of the plasma membrane, occurs during the course of the slow wave.

(6) The EGTA studies suggest the presence of an internal compartment of Ca^{2+} in the vicinity of the contractile proteins, possibly "far" from the plasma membrane. This Ca compartment is released by depolarization of the plasma membrane.

(7) In the longitudinal muscle layer, a population of mitochondria is present within 200Å of the inner surface of the plasma membrane.

(8) Inhibition of mitochondrial energy production results in termination of slow waves but not contractions.

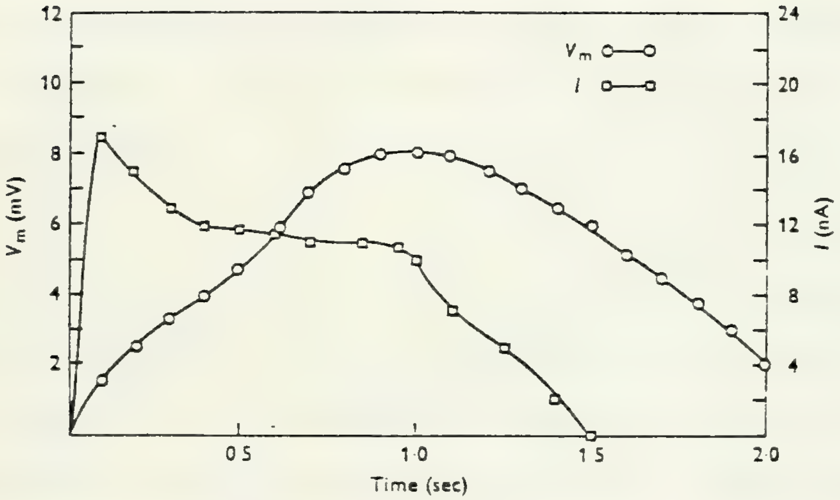
(9) The frequency of NADH oscillations are Ca^{2+} sensitive.

B. Conclusion

As discussed previously (see pg.   ), intestinal slow waves result from the cyclic activity of an electrogenic Na pump. This mechanism, as described in detail by Connor et al. (81) is given below (see Figure 1). During the falling and the diastolic phase of the slow wave, the electrogenic Na pump is operating at a basal level. At a given time, for an unspecified reason, the electrogenic transport rate drops. The decrease in net outward current leaves a net inward leakage current which results in depolarization of the membrane and, therefore, the rising phase of the slow wave. Following the decline in electrogenic Na pump activity the pump level settles to a semi-stable value from which it gradually returns to the basal level. Following the increase in the electrogenic transport rate, the falling phase of the slow wave is produced. Under voltage clamp, a decrease in outward current appears as an inward current. Due to the membrane capacitance the membrane voltage lags the current.

A fundamental, but as of yet unresolved question is, the cause of the Na pump oscillations. Changes in the level of Na pump activity (e.g.,

Figure 1. Simultaneous plot of membrane current and the slow-wave voltage from a single preparation. Inward membrane current is plotted as positive to aid in comparing the two quantities. From Connor _et al._, ref. 81.


V_m (mV)
12
10
8
6
4
2
I (nA)
24
20
16
12
8
4
V_m
I
0.5
1.0
1.5
2.0
Time (sec)

by ouabain or metabolic inhibitors) do not substantially alter the frequency of slow waves (81,232). Thus, the pacemaker reactions cannot be exclusively within the Na pump. As discussed previously (see pgs.), my data are consistent with Ca_i^{2+} modulating the activity of the Na pump. Ca_i^{2+} could, therefore, be involved in the pacemaker process of intestinal slow waves. Given below is a scheme to describe a possible role of intracellular Ca^{2+} in slow wave generation (see Figures 2 and 3).

Since mitochondria may be involved in the process responsible for generation of slow waves, a possible site of Ca^{2+} interaction with the slow wave generation mechanism is the mitochondrial electron transport chain (176,254). During the oxidation of NADH by the electron transport chain, 2 alternative processes can occur: Ca^{2+} transport into the mitochondria or phosphorylation of ADP. Rat liver mitochondria accumulate Ca^{2+} at concentrations down to 1 μM in preference to phosphorylation of ADP. In various smooth muscles, the K_m values reported for Ca^{2+} transport into the mitochondria range from 10^{-7} M to 17 x 10^{-6} M (20,150,240,291). Even in the studies in which the higher K_m values were found, Ca^{2+} was still accumulated by the mitochondria when bathed in solutions containing 1 μM Ca^{2+} (150,240,291).

In order to work through the scheme illustrated in Figure 2, let the Ca^{2+} level bathing the mitochondria involved in slow wave production be below the level which activates path B (i.e., activation level is approximately 1 μM Ca^{2+} for rat liver mitochondria. This level will be referred to as a threshold). As discussed previously (see pg.), the mitochondria located within 200Å of the inner cell membrane may be the mitochondria involved in slow wave generation. Choice of this population has 2 particularly attractive features: (1) these mitochondria are

Figure 2. Model for slow wave generation. See text for a detailed explanation of the scheme.

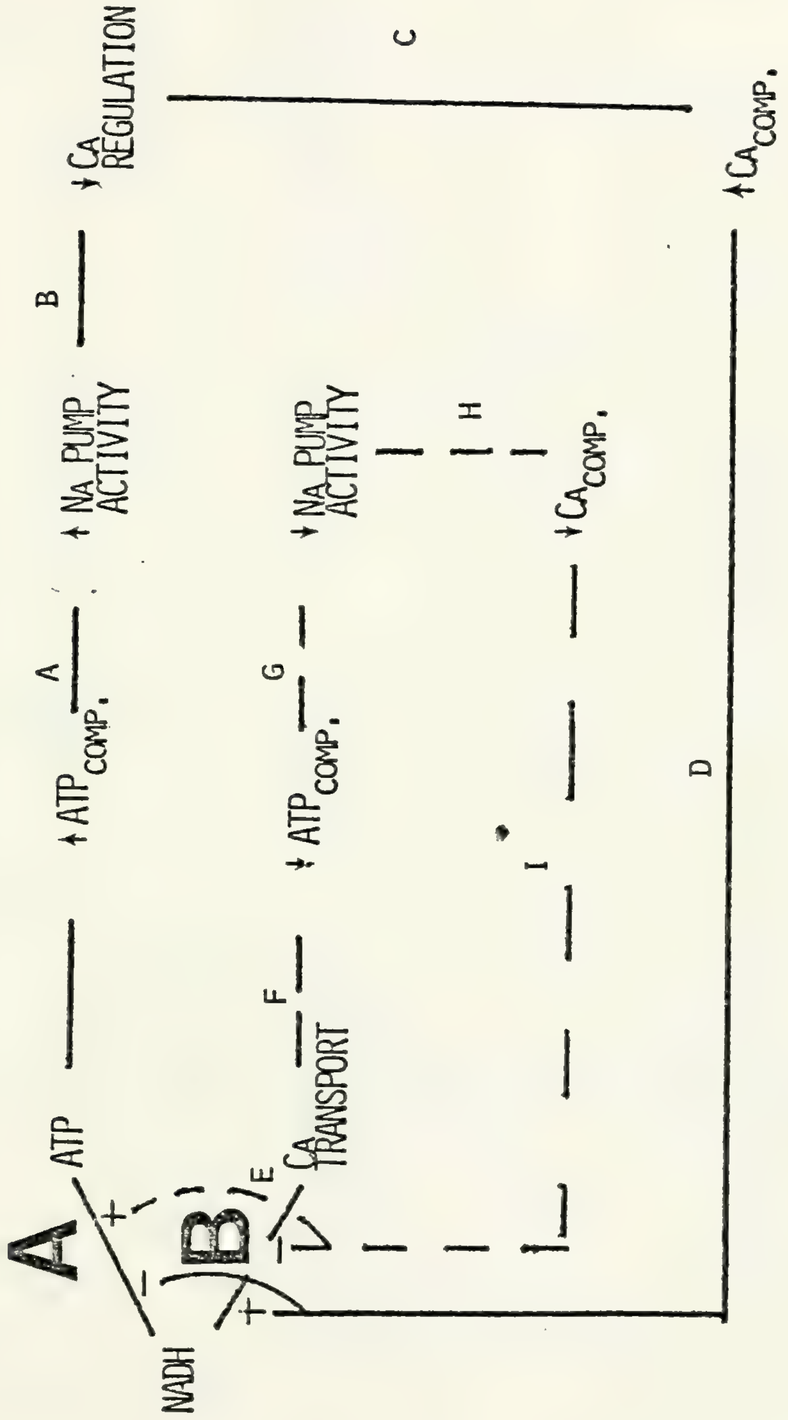
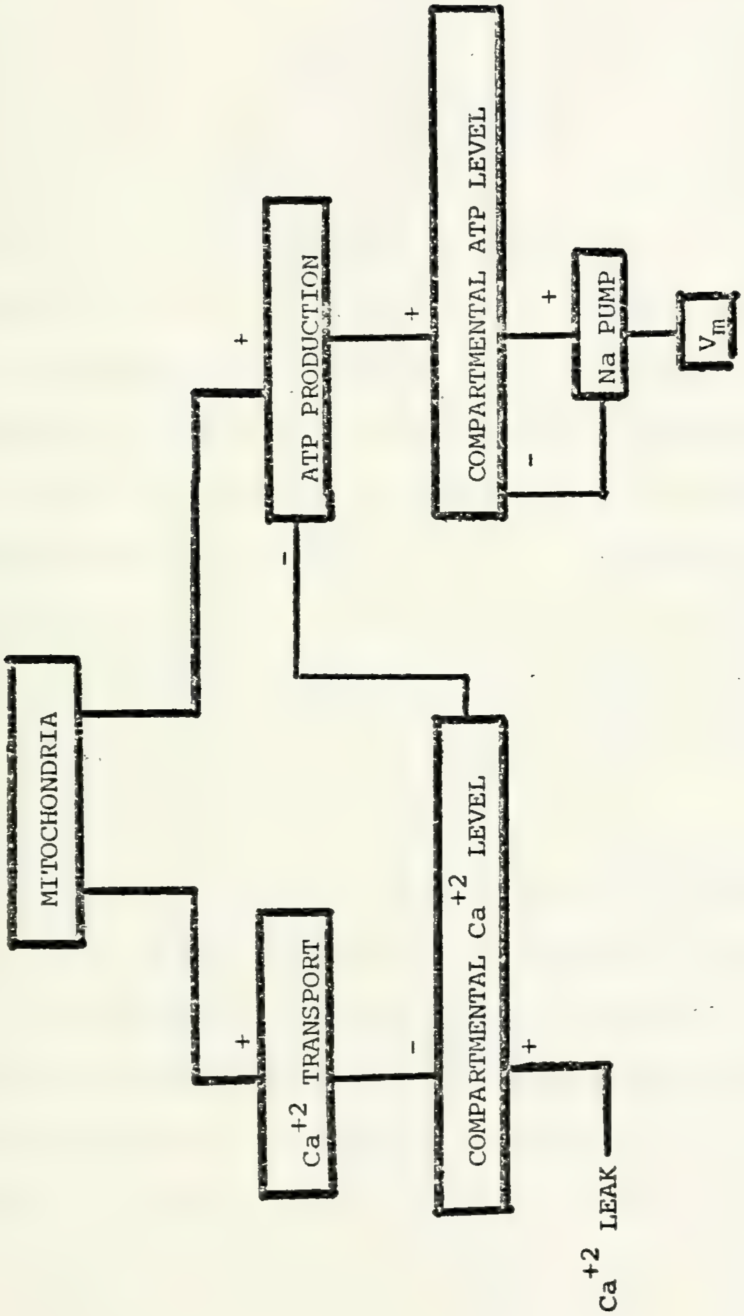

A
B
NADH
ATP
+
−
+
−
E
↓ CA TRANSPORT
F
↓ ATP COMP.
G
↓ NA PUMP ACTIVITY
H
↓ CA COMP.
I
D
↑ ATP COMP.
A
↑ NA PUMP ACTIVITY
B
↓ CA REGULATION
C
↑ CA COMP.

Figure 3. Generalized scheme for slow wave generation.

GENERALIZED SCHEME



preferentially found in the longitudinal muscle layer, the site of slow wave generation. (2) The region between the plasma membrane and the mitochondria is small, less than 200Å. This region may form a compartment in which Ca_i^{2+} and ATP levels are not indicative of the rest of the cell.

Since I am assuming that the level of Ca_i^{2+} bathing the mitochondria is initially below threshold for activation of path B, the switch is in position A and the electron transport chain phosphorylates ADP in preference to transporting Ca^{2+}. During this time, the level of ATP in the compartmental region (i.e., between the cell membrane and mitochondria) is increasing. The increase in compartmental ATP levels produces increases in Na pump activity (step A). Since Ca^{2+} regulation by the mitochondria is now operating at a low level (step B) and because of the continual leak of Ca^{2+} into the cell, compartmental Ca^{2+} content increases (step C). During this time the Na pump has been operating at increased levels; hence the slow wave is in its diastolic phase.

Eventually the level of compartmental Ca^{2+} surpasses the threshold for activation of path B and path B is activated (step D). During this time, the mitochondria transport Ca^{2+} in preference to phosphorylation of ADP (step E) and compartmental ATP levels, therefore, decline (step F). The decline in ATP levels produces a reduction in Na pump activity (step G). Hence, the rising phase of the slow wave is produced. Compartmental Ca^{2+} is eventually sequestered to a level below the threshold for path B (step H) and the cycle is shunted back to path A (step I). Other organelles, particularly the sarcoplasmic reticulum, may aid in fine tuning of compartmental Ca^{2+} levels.

Since the cycling frequency between paths A and B is triggered by a rise or fall in compartmental Ca^{2+} levels, changes in compartmental

Ca^{2+} concentrations should produce changes in slow wave frequency. During periods when the compartmental Ca^{2+} level is decreased, the cycle would be in path A for prolonged periods of time. This can be correlated with an increase in Na pump activity and therefore, prolongation of the diastolic phase of the slow wave. This correlates with my experimental observations during periods of decreased Ca_i^{2+}. The reverse situation would apply for increases in Ca_i^{2+}.

A computer simulation of this scheme was done by Dr. M. Mangel. The simulation shows nondamping oscillations in membrane potential with accompanying oscillations in compartmental Ca^{2+} levels (Figure 4). The frequency of the simulated slow waves is Ca^{2+} sensitive (Figure 5). The diastolic phase of the slow waves is prolonged under conditions of low Ca^{2+} and the systolic phase prolonged following increases in Ca^{2+}.

Figure 4. Computer simulation of scheme in Figure 1. Upper trace; computer simulated voltage represented as a function of ATP levels. When ATP levels are high, the membrane potential is hyperpolarizing. Lower trace; computer simulation of Ca_i^{2+} changes during the course of the simulated slow wave. Computer modeling was graciously done by Dr. Marc S. Mangel (see ref. 190).

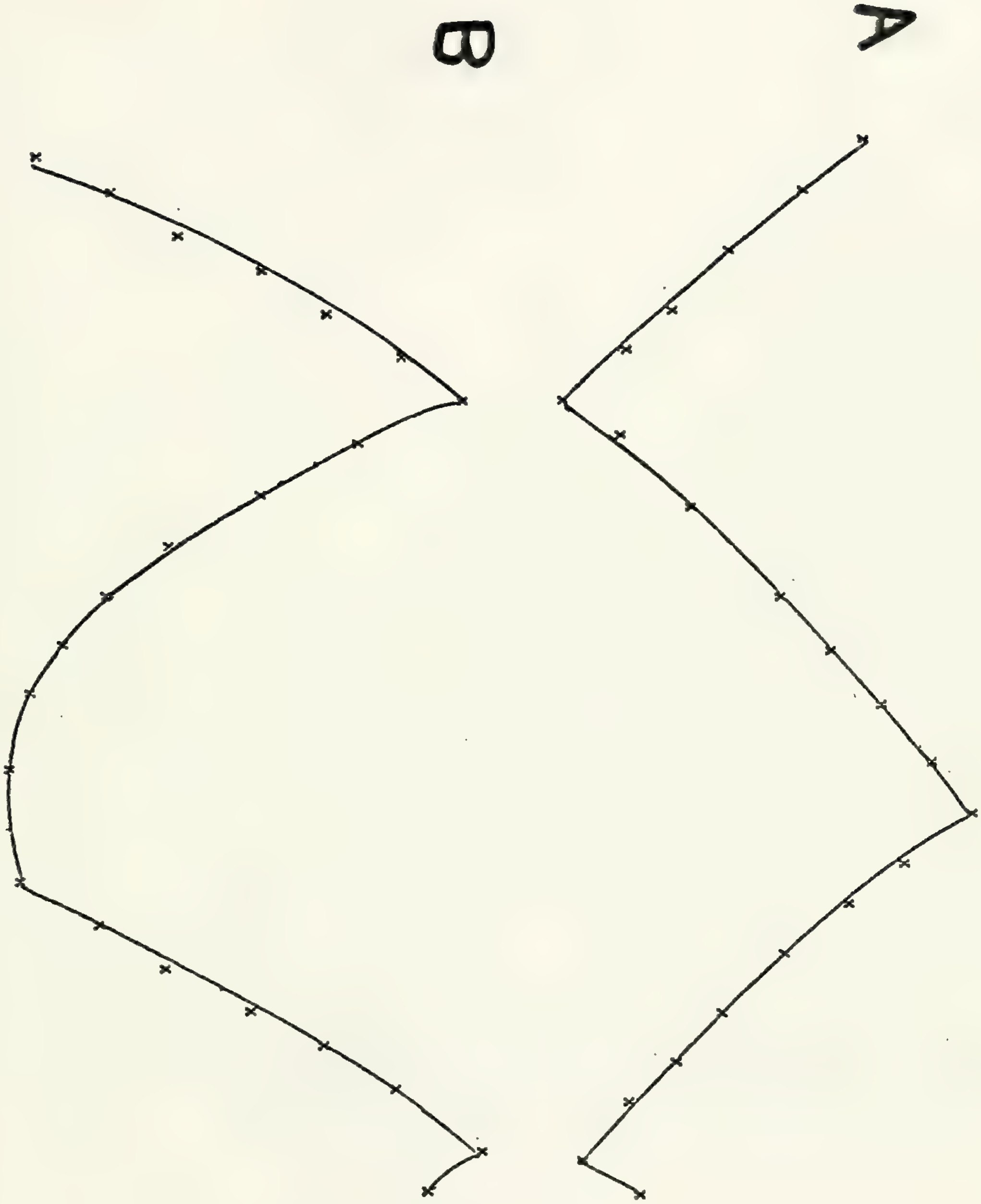

B
A

Figure 5. The effects of altered compartmental Ca^{2+} levels on simulated slow waves. Solid line represents slow wave simulated with normal Ca^{2+} levels. Dotted line is following reduction of Ca^{2+} by a factor of 2 and dashed line represents a doubling of Ca^{2+}.

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Appendix

Abbreviations:

ACh - acetylcholine

Ca - total calcium

Ca^{2+} - ionized calcium

CTC - chlorotetracycline

DNP - dinitrophenol

IAA - iodoacetic acid

Me Blue - methyline blue

MOPS - morpholinopropane sulfonic acid

TEA - tetraethylammonium chloride

TTX - tetrodotoxin

Appendix (by Dr. M. Mangel)

Numerical Simulation of Slow Wave Oscillations

Two systems of equations were used in this simulation (see page for parameters).

System I (for $x < 1$)

$$\dot{x} = A_1 - A_2x + A_3y$$

$$\dot{y} = B_1 - B_2y - \left(\frac{B_3y + B_4y^2}{B_5 + y^2}\right)$$

System II (for $x \geq 1$)

$$\dot{x} = A_1 - A_2x + A_3y - A_4x/1 + A_5x$$

$$\dot{y} = -\left(\frac{B_3y + B_4y^2}{B_5 + y^2}\right)$$

When the value of x crosses 1 from below, there is a delay of Δ_1 before the system II equations take over when the value of x crosses 1 from above, there is a delay of Δ_2 before the system 1 dynamics take over.

The systems of equations I and II, were solved on a Wang 2200 programmable calculator using either a forward difference scheme or a fourth order Runge-Kutta scheme yielding equivalent results in either case. The step size, h, was picked so that $\Delta_1/h >> 1$ and $\Delta_2/h >> 1$. The simulation was obtained according to the following alogarithm.

(1) Enter $(x(o), y(o))$. Set $t=0$.

(2) If $x(t) > 1$ go to step 6.

(3) If $x(t - \Delta_2) > 1$, go to step 5.

(4) (Remark: Get here if $x(t) < \Delta 1$ and $x(t - \Delta_1) < 1$). Increment $(x(t), y(t))$ to $(x(t + h), y(t + h))$ by solving system I. Go to step 9.

(5) (Remark: Get here if $x(t) < 1$ but $x(t - \Delta_2) \geq 1$, so system II dynamics hold). Increment $(x(t), y(t)$ to $(x(t + h), y(t + h)$ by solving system II. Go to step 9.

(6) (Remark: Get here if $x(t) > 1$). If $x(t - \Delta_1) < 1$ go to step 8.

(7) (Remark: Get here if $x(t) > 1$ and $x(t - \Delta_1) > 1$, so system II dynamics hold). Go to step 5.

(8) (Remark: Get here if $x(t) > 1$ and $x(t - \Delta_1) < 1$, so system I dynamics hold). Go to step 4.

(9) Replace $(x(t), y(t))$ by $(x(t + h), y(t + h)$ and t by $t + h$.

(10) Go to step 2.

The parameters used are given below.

t = time

x = Ca^{2+} concentration

y = ATP concentration

$\dot{x}$ = change in Ca^{2+} concentration

$\dot{y}$ = change in ATP concentration

$A_1 = 10$

$A_2 = .1$

$A_3 = 1$

$B_1 = 2$

$B_2 = 1$

$B_3 = 20$

$B_4 = .1$

$B_5 = 20$

$A_4 = 40$

$A_5 = 1$

Vita

Allen Wayne Mangel was born January 17, 1953 in New York City, New York. He attended Highland Park High School in Highland Park, Illinois. He obtained a B.S. degree in Physiology from the University of Illinois in 1974. He obtained a M.S. degree in Physiology from the University of Illinois in 1977 and Ph.D. degree in Physiology in 1980. While in graduate school Allen was a teaching assistant from 1974-1977, research assistant from 1977-1978 and NIH trainee from 1978-1980.

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